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Molecular Regulation of Embryonic Mammary Gland Development

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ACADEMIC DISSERTATION

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Somewhere, something
incredible is waiting to be known.

-Carl Sagan

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III **Voutilainen M**, Lindfors PH, Lefebvre S, Ahtiainen L, Fliniaux I, Rysti E, Murtoniemi M, Schneider P, Schmidt-Ullrich R, Mikkola ML. 2012. *Ectodysplasin regulates hormone-independent mammary ductal morphogenesis via NF-κB*. Proceedings of the National Academy of Sciences 109(15):5744-9.

This thesis is based on original publications, which in the text are referred to by their Roman numerals

Abbreviations

ADAMTS	A Dissintegrin And Metalloproteinase with Thrombospondin Motifs
AR	Androgen receptor
AREG	Amphiregulin
BMP	Bone morphogenetic protein
DKK	Dickkopf
E	Embryonic day
ED	Ectodermal dysplasia
EDA	Ectodysplasin, Eda-A1
EDAR	Ectodysplasin A1 receptor
EDARADD	Edar-associated death domain
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EPGN	Epigen
FGF	Fibroblast growth factor
GLI	Glioma-associated oncogene homolog
HED	Hypohidrotic ectodermal dysplasia
HH	Hedgehog protein family
IκB	Inhibitor of kappa B
IKK	IκB kinase complex
ISH	In situ hybridization
JNK	c-Jun N-terminal kinase
K14	Keratin-14 promoter
KRM	Kremen, kringle Containing Transmembrane Protein
LEF-1	Lymphoid enhancer-binding factor 1
LGR	Leucine-rich repeat-containing G protein-coupled receptor
LRP	Low-density lipoprotein receptor-related protein
MAD-CAM	mucosal vascular addressin cell adhesion molecule
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
MSX	vertebrate homolog of Drosophila muscle segment (Msh) homeobox gene
NF-κB	Nuclear factor kappa-B
NEMO	NF-κB Essential Modulator (=IKKγ)
NRG	Neuregulin
PTHRP	Parathyroid hormone-related protein
RANKL	Receptor activator of Nf-κb-ligand
R-SPONDIN	Reelin domain-containing spondin
RISH	Radioactive in situ hybridization
RT-PCR	Reverse transcriptase polymerase chain reaction
SHH	Sonic hedgehog
SOSTDC1	Sclerostin domain-containing 1
TGFβ	Transforming growth factor β
TNF	Tumour necrosis factor

TNFR	Tumour necrosis factor receptor
TRAF	TNF-receptor associated factor
TBX	T-box
XEDAR	Ectodysplasin A2 receptor
XLHED	X-linked hypohidrotic ectodermal dysplasia
WMISH	Whole mount in situ hybridization
WNT	Wnt- family member
WT	Wild type

Summary

Mammary gland development begins during embryogenesis with the formation of species-typical number of mammary placodes that emerge along the flanks of the embryo at conserved positions. By birth, the mammary primordium has undergone branching morphogenesis and displays a small ductal tree with several branches. The organ development and growth continues throughout postnatal life and the mammary gland matures to functional form only during pregnancy and following lactation. Ectodysplasin (Eda), a member of the tumour necrosis factor family, is one of the key regulators of epithelial appendage development in all vertebrates. In humans, mutations in the *Eda* gene, or in other components of the signalling pathway, cause hypohidrotic ectodermal dysplasia (HED), a disorder characterized by sparse hair, missing teeth, and defects in several exocrine glands including the breast. Previous studies have shown that transgenic overexpression of *Eda* (K14-*Eda* mice) in the developing ectoderm leads to formation of ectopic mammary placodes, which give rise to supernumerary glands in the adult mice. Otherwise, effects of Eda signalling in the mammary gland have been fairly unknown.

Here I have analysed the role of Eda in prepubertal mammary gland development. Characterization of the mammary glands of *Eda* gain- (K14-*Eda*) and loss-of-function (*Eda* $^{-/-}$) mice revealed that the branching morphogenesis of the organs correlated with Eda levels. Overexpression of Eda induced precocious and accelerated branching whereas lack of Eda reduced number of ductal tips. Furthermore, Eda induced supernumerary mammary placode formation not only on the flank but also in the neck region. Analysis of the mouse line with suppressed NF-κB signaling (*IκBαΔN* mice) revealed that the transcription factor is a major mediator of Eda in the mammary gland. NF-κB activity was shown to be necessary for the ability of Eda to induce supernumerary mammary primordia and to accelerate branching morphogenesis. With a candidate gene approach and genome wide-profiling several potent Eda target genes were identified in the mammary gland. Among them were members of the Wnt/β-cat pathway. The obtained results suggest that Eda promotes mammary cell fate by enhancing canonical Wnt pathway activity and other effects of Eda are cooperatively mediated by certain Wnt family members in addition to other factors. To study mammary placode formation and branching morphogenesis and to assess roles of individual downstream factors or pathways, *ex vivo* culture systems were developed and utilized in this thesis work.

1 Review of the literature

1.1 Mammary gland as an ectodermal organ

Mammary glands are ectodermal organs, a group of epithelial appendages that include many exocrine glands such as salivary and sweat glands along with a large repertoire of other skin derivatives like hair, teeth, scales, feathers, nails and horns. Induction and early growth of the organs during embryogenesis involves highly similar morphological aspects even though their mature form and purpose varies enormously (Biggs and Mikkola, 2014). The first sign of morphogenesis is the appearance of a local lens-shaped epithelial thickening referred to as a placode, which is a shared feature in the early development of all ectodermal appendages. Invagination of the placode into the underlying mesenchyme produces a bud. Sequential growth followed by organ-typical branching or folding of the epithelium gives rise to distinctive structures such as the small, branched ductal tree of a nascent mammary gland (Biggs and Mikkola, 2014;Howard, 2012).

The same molecular pathways have been co-opted to function in the organogenesis of several ectodermal organs, which in part is thought to reflect their common evolutionary history. Dysfunction of many signalling pathways may lead to defects in multiple epithelial appendages, a condition called ectodermal dysplasia (Itin, 2014). Sequential and reciprocal interaction between the epithelium and the underlying mesenchyme is essential for proper development of all ectodermal organs (Biggs and Mikkola, 2014;Howard, 2012).

1.1.1 Branching morphogenesis

Branching morphogenesis is a frequently utilized developmental program to achieve a large surface area in a wide spectrum of organs. It is typical to some but not restricted to ectodermal organs. Many structures, ranging from invertebrate's tracheal system to vertebrate mammary gland and lung, accomplish extensive exchange or production of substances in an organ of considerably small volume compared to its total epithelial surface area. Despite the unique characteristics of the organs, a conserved set of molecular regulators are utilized in their morphogenesis. In most cases, successful epithelial development relies on supportive and sustaining signals or chemo-attractant cues that originate from the surrounding mesenchyme (Lu and Werb, 2008;Varner and Nelson, 2014).

Branch pattern refers to organ-specific features such as branch initiation point and length and diameter of the ducts. In the lung the branch pattern is stereotypic and is governed by tight genetic regulation, whereas in a mammary gland the induction of specific branches appears not to be predetermined. Inhibitory effects of the pre-existing tips for example are thought to prevent induction of new branches in close proximity of the existing ones during pubertal branching morphogenesis (Lu and Werb, 2008).

1.2 Molecular regulation of morphogenesis and signalling pathways involved in mammogenesis

Cells of an organism do not live in isolation but instead are in constant interaction with each other. Cell populations that carry out specific functions and have similar origins give rise to tissues that in turn join together to form the organs of an individual. Cell and tissue diversity is achieved in stages through differentiation from nascent to more committed cells. It involves

constant communication between individual cells and tissue compartments, which is also a requisite for maintaining homeostasis later on.

Cells communicate via various signalling molecules that form pathways which in turn form more complex networks. Commonly a signalling molecule is a ligand that binds to a specific receptor, which triggers intracellular signalling cascades to exert the message. Signalling cascades are typically controlled in multiple phases by various negative or positive regulators that can amplify or inhibit the signal transduction. Often the cellular response is an activation of transcription factors that bind to the enhancer or promoter regions of a downstream gene. This leads to modulation of gene expression that can be either suppressive or activating. Eventually the received signal is either forwarded to other cells or interpreted as a change in cellular processes such as proliferation, apoptosis, cell shape changes or migration.

Many signalling molecules are soluble, which allows them to diffuse to the surroundings. The regulatory molecules can be sensed by nearby cells, referred to as paracrine signalling, or affect the signal-producing cell in an autocrine manner. For example the epithelial-mesenchymal crosstalk during ectodermal organ development relies on soluble signalling factors acting via paracrine signalling. Additionally, cell interaction can also occur via direct cell-cell contact (Biggs and Mikkola, 2014;Gilbert, 2010).

During development multiple signalling molecules are typically co-produced by transitory signalling centres. The mammary placode for instance expresses several signalling molecules that can have either activating or inhibiting effects on the placode itself or on the surrounding area. According to the reaction-diffusion model these factors can have unequal diffusion and different stabilities. This is thought to enable placode-promoting factors to be maintained in a higher level within the structure and inhibitors to diffuse further away, which localizes the placode-promoting cues to the developing organ primordia. This in turn is necessary for correct spatial patterning of the organs (Biggs and Mikkola, 2014;Kondo and Miura, 2010).

The competence of cells to receive, interpret and respond to the signals from the microenvironment is vital for proper development as well as for maintaining homeostasis of a system or an organ. Faulty information-processing by the cell can lead to defects in normal development or be responsible for the occurrence of diseases such as cancer (Gilbert, 2010).

1.2.1 Fibroblast growth factor pathway

Fibroblast growth factor (Fgf) family proteins bind to specific receptor tyrosine kinases. In mammals Fgf receptors (Fgfr) are encoded by four genes (*Fgfr1-4*). Fgfr1–Fgfr3 can undergo alternative splicing to generate two distinct isoforms, b and c (Itoh and Ornitz, 2011). *In vitro* studies have shown that Fgfs do not bind exclusively to one receptor type but interact with several different Fgfrs with varying affinity. Fgfrs signal as dimers that are formed upon ligand binding. Stimulation of the pathway leads to a series of intracellular phosphorylations that trigger four distinct signal transduction pathways: MAPK, PI3K, STAT and phospholipase Cγ (Turner and Grose, 2010).

1.2.2 Wnt pathway

Wnt ligands are secreted signalling molecules that bind to a receptor complex comprising a transmembrane Frizzled receptor and co-receptor Lrp5 or Lrp6. To date Wnts have been described to activate three distinct pathways (Komiya and Habas, 2008). Since the non-canonical planar cell polarity and calcium-mediated pathways have, thus far, not been shown to be

essential for prepubertal mammary gland development (Boras-Granic and Hamel, 2013), they will not be described here further. The canonical Wnt/β-catenin (Wnt/β-cat) pathway regulates intracellular β-cat levels and activity of the Lef1/Tcf transcription factors that are kept inactive in unstimulated cells by a specific repressor protein. In the absence of ligand a multiprotein destruction complex consisting of Axin, CK1, GSK3β, and APC, ubiquitylates cytoplasmic β-cat which becomes proteolytically degraded. Receptor complex activation leads to inhibition of β-cat phosphorylation. This enables its cytoplasmic accumulation and translocation to nucleus. As β-cat binds to Lef1/Tcf proteins it displaces the repressor protein and the newly formed complex is transcriptionally active (Niehrs, 2012). R-spondins are secreted Wnt agonists and ligands of Lgr receptors (Lgr4-6). Though R-spondins do not initiate Wnt signalling, they potentiate it (Cruciat and Niehrs, 2013;de Lau et al., 2012).

1.2.3 Transforming growth factor β pathway

The transforming growth factor beta (Tgf-β) family consists of several members, including the bone morphogenetic proteins (Bmp). Tgf-β ligands activate their receptors as dimers. The signalling pathway receptors are heterodimeric complexes that compose type I and type II subunits with serine/threonine kinase domains. Ligand-activated receptors eventually initiate a Smad-dependent signalling cascade (Kamato et al., 2013;Massague, 2000).

1.2.4 Epidermal growth factor pathway

The epidermal growth factor (Egf) family includes proteins such as Egf itself, Amphiregulin (Areg) and Epigen (Epgn) as well as Neuregulin (Nrg) family members. These molecules activate transmembrane tyrosine kinase receptor ErbB proteins. Egf, Areg and Epgn signal through ErbB1 (Egfr) whereas Nrgs bind to the ErbB3 and ErbB4 receptors. ErbB2 does not bind directly to ligands but instead is in a conformation that resembles the ligand-activated state. Activated receptors undergo dimerization and phosphorylation that ultimately triggers intracellular PI3K and MAPK pathways (Arteaga and Engelman, 2014;Olayioye et al., 2000;Wilson et al., 2012).

1.2.5 Sonic hedgehog pathway

Sonic hedgehog (Shh) is a member of the Hedgehog (Hh) family. In canonical Hedgehog signalling, transmembrane proteins Patched (Ptch) and Smoothened (Smo) mediate signal transduction. In the absence of a ligand Ptch represses Smo. Hh binding to Ptch relieves its inhibitory action on Smo, which allows further signal transduction by the Gli family of transcriptional activators and repressors (Gli1-Gli3). Gli1 encodes transcriptional activator, whereas Gli2 and Gli3 are activators of the pathway in the presence of positive Hh signalling but are cleaved to render transcriptional repressors in the absence of Hh (Sasai and Briscoe, 2012).

1.2.6 Parathyroid hormone-related protein pathway

Parathyroid hormone-related protein (Pthrp) is encoded by the Pthlh gene. It is structurally related to a parathyroid hormone commonly known as a regulator of circulatory calcium levels. Pthrp binds a distinct G protein-coupled receptor Pthr1. The receptor signals via several different intracellular secondary-messenger systems to regulate target gene expression (Vilardaga et al., 2011;Wysolmerski, 2012).

1.3 Ectodysplasin

Ectodysplasin (Eda) belongs to the tumour necrosis factor (Tnf) superfamily. *Eda* gene gives rise to two biologically active splice variants, Eda-A1 and Eda-A2 that signal through distinct receptors, Edar and Xedar, respectively (Yan et al., 2000). Eda-A1 (hereafter Eda) has a well-characterized role in the morphogenesis of several ectodermal organs, whereas the physiological function of Eda-A2 is poorly understood (Lefebvre and Mikkola, 2014) and thus will not be discussed here further.

1.3.1 Eda signal transduction

Eda is a trimeric type II membrane protein. The extracellular portion consists of a cleavage site for furin enzyme, a collagen domain and the C-terminal TNF homology domain that is responsible for receptor binding. Release from the cell surface is required for the biological activity of Eda. Upon proteolytic cleavage by furin, Eda is shed from the cell membrane, which allows its diffusion to the site of transmembrane receptor Edar (Elomaa et al., 2001). Ligand-stimulated Edar interacts via intracellular adapter protein Edaradd which links the receptor to the downstream signalling cascade (Figure 1). Several lines of evidence have shown the role of the transcription factor nuclear factor-kB (NF-κB) as an important downstream effector of Eda (Mikkola, 2009), but other downstream mediators, in particular the c-Jun N-terminal kinase (JNK) have also been implicated (Kumar et al., 2001). In unstimulated cells, NF-κB is retained in the cytosol by IκB inhibitory proteins, most commonly by IκBα. Upon ligand binding, Edaradd becomes associated with Trafs (Tnfr-associated factors), mainly Traf 6. Recruitment of Tab2 (Tak1-binding protein 2) and Tak1 (Tgfβ-activated kinase 1) to the signalling cascade mediates the activation of IκB-kinase (IKK) complex, which is composed of two catalytic subunits, IkappaB kinase alpha (IKKα or Ikk1) and beta (IKKβ) and a regulatory protein NF-kappa-B essential modulator (Nemo or IKKγ). IKK complex phosphorylates the IκB inhibitory proteins (Morlon et al., 2005;Perkins, 2007), which leads to their subsequent degradation and releases NF-κB for nuclear translocation, thereby permitting transcriptional regulation of the target genes (Perkins, 2007).

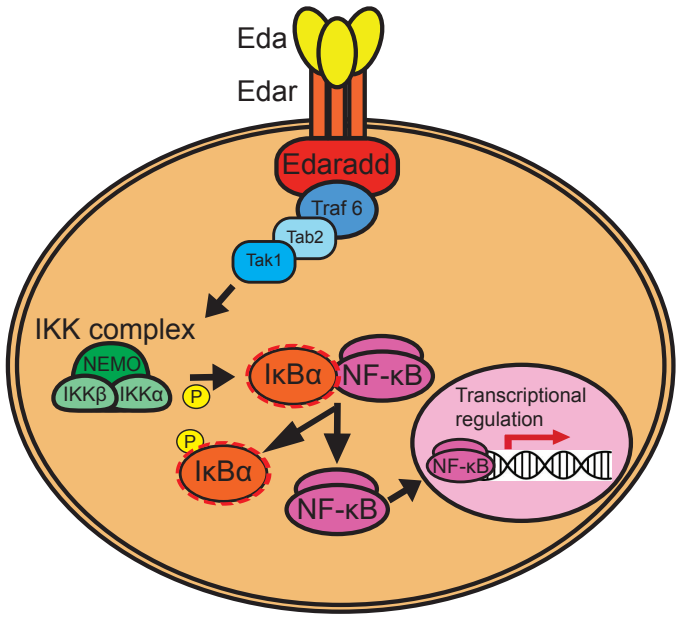

Figure 1. The Eda signalling pathway.
In unstimulated cells NF-κB is bound to IκB (mostly IκBα) inhibitory proteins. Ligand binding to Edar receptor initiates the Edaradd-mediated intracellular signalling cascade. Activated IKK complex phosphorylates IκBs that become proteolytically degraded. This enables nuclear translocation of NF-κB.

The mammalian NF-κB family includes five structurally related transcription factors (p65 or RelA, RelB, c-Rel, p50/p105 and p52/p100) (Gilmore, 2006;Verma et al., 1995). Their shared sequence homology is responsible for DNA binding, dimerization of the factors and association with the IκB inhibitory proteins. NF-κB can be activated by two distinct pathways. The canonical pathway described above, which operates with dimers composed of RelA or c-Rel, and p50, has been implicated in Eda signalling (Kowalczyk-Quintas and Schneider, 2014). The alternative, non-canonical pathway does not require the IKK complex but instead relies only on IKKα. IKKα activation leads to protelolytic processing of p100 to p52 which typically associates with RelB to regulate transcription (Scheidereit, 2006).

1.3.2 Eda deficiency and Hypohidrotic ectodermal dysplasia

The Eda signalling pathway is vital for ectodermal organogenesis and largely conserved in all studied vertebrate species. Disruption of Eda signalling causes hypohidrotic ectodermal dysplasia (HED), which leads to defective ectodermal appendage formation even across taxa (Drew et al., 2007;Harris et al., 2008;Kondo et al., 2001;Mikkola, 2009).

Mutations in the murine X-chromosomal *Eda*, and autosomal *Edar* or *Edaradd* have been identified in mouse models *Tabby* (*Eda* null, hereafter *Eda* $^{-/-}$) *Downless, Sleek* (*Edar* deficient) and *Crinkled* (*Edaradd* deficient) that share highly similar or nearly identical phenotypes (Falconer, 1952;Gruneberg, 1971;Headon et al., 2001;Sofaer, 1969;Yan et al., 2002). The *Eda* $^{-/-}$ is the most extensively studied of them. Characteristic features of these mice include the lack

or malformation of dentition and defects in hair development. Several exocrine glands are also affected to varying degrees (Gruneberg, 1965;Gruneberg, 1971;Mikkola, 2009). Mutation of *Traf6* gives rise to a remarkably similar ectodermal phenotype, as does the loss of NF-κB activity in a mouse strain that expresses a non-degradable form of the IκBα inhibitory protein (Naito et al., 2002;Schmidt-Ullrich et al., 2001). Similarities between the different loss-of-function phenotypes further indicate a mutual dependency of the factors in Eda pathway activation.

In humans the X-linked hypohidrotic ectodermal dysplasia (XLHED) is the most common form of a vast spectrum of nearly 200 ectodermal dysplasias (Kere et al., 1996;Visinoni et al., 2009). The most typical features are sparse hair and missing or malformed teeth. Additionally many exocrine glands, such as salivary and sweat glands, among others, are absent or function inadequately, which leads to reduced secretion of saliva and heat intolerance due to the inability to perspire. Especially affected children are prone to perilously high fever due to the impaired ability to sweat (Mikkola, 2009). Anomalies of the breasts, though reported occasionally, have thus far not been documented to the same extent as other developmental deficiencies (Clarke et al., 1987).

Prevalence of XLHED in human population has been estimated to range from 1:4500 to 1:62000 (Nguyen-Nielsen et al., 2013). The phenotypically similar but autosomally inherited form of HED is caused by defects in the genes coding for the other members of the signalling pathway; receptor Edar and adaptor protein Edaradd (Headon and Overbeek, 1999;Headon et al., 2001;Srivastava et al., 1997). Additionally hypomorphic mutations in *NEMO* or *IκB*α lead to HED accompanied by an impaired immune system (Courtois et al., 2003;Doffinger et al., 2001;Zonana et al., 2000). As the *EDA* gene is X-chromosomal, the syndrome is most often reported in men, whereas women are commonly carriers of the condition. The relatively rare autosomal forms affect both sexes equally and can be either dominant or recessive. Female carriers of XLHED and all patients with autosomal dominant HED usually display milder forms of the disease and often exhibit only slight manifestations of the cardinal traits (Mikkola, 2009).

Beyond managing the symptoms, no treatment of the condition is commonly available. However, introduction of recombinant Eda protein engineered to cross the placental barrier to *Eda*$^{-/-}$ female mice during gestation permanently corrects many of the defects (Gaide and Schneider, 2003). Similar correction of the phenotype has been achieved in a dog model for HED with administration of the protein during the first postnatal weeks (Casal et al., 2007; Mauldin et al., 2009). The first clinical trials on new-born babies to compensate for the absent EDA protein with a pharmacological substitute have been ongoing since 2013 (Huttner, 2014).

1.3.3 Eda gain-of-function mouse models

In contrast to lack of Eda, increased Eda levels stimulate ectodermal organ development. K14-*Eda* mice overexpress *Eda* under the K14-promoter which drives expression of the transgene into the ectoderm and later to the basal layer of the skin from mid-gestation (embryonic day 9.5, E9.5) onwards. The mouse model has alterations in the shape and number of several skin appendages already during embryonic morphogenesis (Mustonen et al., 2003). Typically the hair and tooth placodes are enlarged. Additionally, the mice develop an ectopic molar placode in the oral epithelium and usually two to three supernumerary mammary placodes within the mammary-forming region. At least some of these accessory structures gradually transform into rudimentary organs present in the adult animal (Kangas et al., 2004;Mustonen et al., 2004). Furthermore, embryonic salivary glands display accelerated branching morphogenesis (Häärä et

al., 2011). Adult mice have increased sweat production, enlarged sebaceous glands, altered hair composition and higher complexity of endogenous dentition (Kangas et al., 2004;Mustonen et al., 2003;Mustonen et al., 2004).

In addition to K14-*Eda* mice, another gain-of-function mouse model has been generated. It harbours multiple copies of the entire *Edar* locus (*Edar*^{TG951}). These mice have highly-branched mammary glands in adolescence. Also other exocrine glands are affected. The sebaceous and meibomian glands are enlarged and the salivary glands exhibit more elaborate branching (Chang et al., 2009). Whether the observed phenotypes are a result of altered embryonic development or caused by later morphological changes was not studied.

1.3.4 Natural variation in Eda signalling

Ectodermal appendages display variation in density and morphology within different populations of a species. As various skin derivatives are at the forefront between an individual and environment, the morphological evolution of the organs provides the means for adapting to a particular habitat. An alternative *Eda* allele has been shown to give rise to the differences in the body armour between stickleback fish in either marine or fresh water surroundings (Colosimo et al., 2005). In modern human populations, an *EDAR* gain-of-function coding variant (V370A) has been associated with a thicker hair phenotype, altered tooth morphology, and increased sweat gland density in east Asia (Fujimoto et al., 2008;Kamberov et al., 2013;Kimura et al., 2009;Mou et al., 2008).

1.3.5 Expression of Eda pathway components

Prior to placode induction *Eda* and *Edar* are co-expressed in the ectoderm. Upon placode formation, *Edar* expression becomes strictly localized to the emerging primordia whereas Eda has been detected as a broader signal in the adjacent epithelium. During salivary gland development, prominent *Eda* expression has instead been found in the mesenchyme (Häärä et al., 2012;Headon and Overbeek, 1999;Laurikkala et al., 2001;Tucker et al., 2000). As development progresses, the expression of these genes eventually overlapps in certain parts of hair follicles (Laurikkala et al., 2002;Pispa et al., 2008). In developing mammary rudiments, *Edar* is found in the mammary epithelium, which is also characterized by high NF-κB activity (Bhakar et al., 2002;Pispa et al., 2003;Pispa et al., 2008). Yet, the expression of *Eda* in a mammary gland context has not previously been described.

In vitro evidence suggests that Eda is induced by the Wnt pathway (Laurikkala et al., 2002). To support this, *Eda* is downregulated in *Lef1* deficient mice and an active Lef1 binding site has been located in the *Eda* promoter (Durmowicz et al., 2002;Laurikkala et al., 2002). Moreover *Edar* expression is likewise upregulated by Wnt/β-cat and according *to in vitro* studies also by Activin. Previous studies have also shown *Edar* to be positively regulated by its own expression whereas Bmp4 signalling downregulates the receptor in the interplacodal area thus restricting *Edar* expression to the placodes. (Laurikkala et al., 2002;Mou et al., 2006;Zhang et al., 2009).

1.3.6 Molecular targets of Eda

Over the years, putative Eda/NF-κB target genes have been uncovered by candidate gene approaches (Mou et al., 2006;Pummila et al., 2007). Genome-wide approaches have compared transcriptomes of wild type and *Eda* gain and loss-of-function mice (Cui et al., 2002;Cui et al., 2006) and more recently, embryonic *Eda* $^{-/-}$ skin exposed to recombinant Eda protein (Fliniaux

et al., 2008;Lefebvre et al., 2012). In general, the pathway has been studied more extensively in hair and teeth. Therefore many transcriptional targets have been identified in these organs, whereas the genes mediating Eda signalling in other organs such as mammary glands remain largely unknown. As several signalling pathways are shared between different ectodermal organs, it would however be expected that Eda target genes are also shared at least to some extent, though organ-specific differences are bound to exist.

To date, the Eda pathway has been shown to interact with multiple major pathways involved in morphogenesis. For instance, Eda controls a group of Wnt pathway genes. Agonists *Wnt10a*, *Wnt10b* (Lefebvre et al., 2012;Zhang et al., 2009) and antagonist *Dkk4* (Fliniaux et al., 2008), are upregulated by Eda in hair placodes. In addition expressions of *Shh*, C*tgf (connective tissue growth factor), Follistatin* (Pummila et al., 2007), several chemokines (Lefebvre et al., 2012), *Fgf20* (Huh et al., 2013), *Foxi3* (Shirokova et al., 2013) and *Edar* (Mou et al., 2006) are modulated by Eda signalling during hair development. *Fgf20* is additionally induced by Eda in salivary glands (Häärä et al., 2012) and *Foxi3* in numerous other foetal skin appendages (Shirokova et al., 2013).

1.4 Mammary gland development

Mammary glands are the most characteristic feature of all mammals. Although the detailed structure, number and location vary between species (Bateson, 1894), they are always composed of a branched epithelial ductal tree and mesenchymal stroma. The stroma that comprises mainly adipose tissue, fibroblasts and a lesser amount of other cell types supports the organ's proper development and maintains it throughout life. The successful growth and function of the mammary glands is vital for the survival of offspring since the secreted milk is the main nutritional source of a new-born. Moreover, the produced milk strengthens the infant's immune system by providing factors such as immunoglobulins and antibodies. Beyond its primary function, nursing of the young is believed to promote an emotional bond between mother and offspring (Anderson et al., 2007;Koyama et al., 2013;Propper et al., 2013).

Mammary glands are a late evolutionary novelty. Mammary glands are initially thought to have arisen from glandular structures that resembled enlarged sebaceous glands. It has been speculated that they were originally used by the fully terrestrial predecessors of mammals to secrete moisture which protected their parchment-shelled eggs from drying (Howard and Lu, 2014;Oftedal and Dhouailly, 2013).

Mammary gland development can be divided in three distinct growth phases. The embryonic development is characterized by the formation of a species-typical number of mammary primordia and the onset of branching morphogenesis. The majority of the ductal growth and branching occurs during adolescence as the circulating ovarian hormones induce exponential branching of the rudimentary ductal tree. The most profound transition occurs during pregnancy and onset of lactation when hormonal cues guide the mammary gland to mature to its functional form (Cowin and Wysolmerski, 2010;Watson and Khaled, 2008).

1.4.1 Morphological development

From milk line to placode

The murine mammary gland development begins approximately at embryonic day E10.5 with the bilateral formation of milk lines (Figure 2) (Propper et al., 2013). Milk lines, however, are not visible to the naked eye but only obvious from histological sections or by molecular detection of

certain marker genes such as *Wnt10b* (Veltmaat et al., 2004). The pseudostratified ectodermal stripes that express *Wnt10b* arise at the ventrolateral border of the embryo, in the axillary and inguinal region and between the fore and hind limbs (Veltmaat et al., 2004).

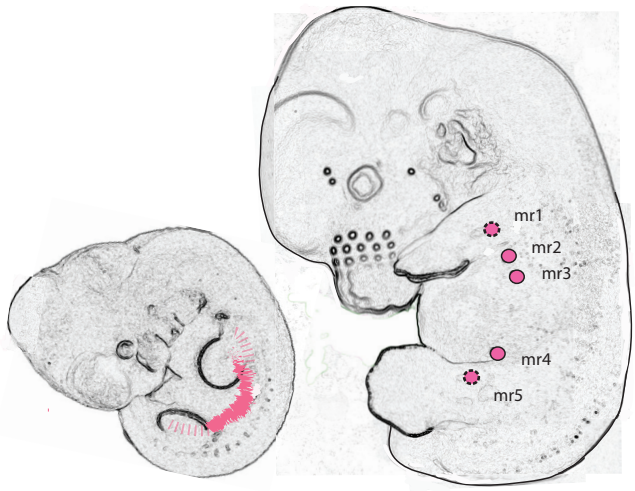

Figure 2. Position of milk lines and mammary rudiments in mice embryo.
The milk lines form bilaterally on the ventrolateral borders of the flanks (left). The central streak ( ) appears between the limbs whereas independent stripes form underneath the limbs ( ). Five mammary rudiments (mr1-mr5) emerge, on both flanks of the embryo, three in the axial and two in the inguinal region (right); (dashed lines indicate rudiments underneath the limbs).

Milk lines have been described as morphologically elevated ridges in certain mammalian species such as the rabbit and human (Propper, 1978;Propper et al., 2013). Recently however, it was pointed out that the ridges found in rabbit may in fact reflect a more advanced stage of development and not the onset of mammogenesis. Similarly to mouse, multilayering of the rabbit milk line precedes the elevation of the structures (Propper, 1978;Propper et al., 2013).

By E11.5 five pairs of mammary placodes, the number of glands in the adult animal, emerge symmetrically at conserved positions along the areas defined by milk lines (Figure 2 and 3). For the sake of clarity, in literature the mammary gland pairs are often numbered from one to five, in rostro-caudal order (Biggs and Mikkola, 2014;Cowin and Wysolmerski, 2010). Placode pair one arises from the axillary milk line; numbers two, three and four from the milk line of the flank, and the inguinal streak gives rise to the fifth placode pair (Veltmaat et al., 2004).

Even though different mammary primordia come across as identical, the appearance of distinct placode pairs is asynchronous, independent from one another and does not occur in an anterior to posterior order typical to other repetitive structures of the body. Rudiment number three is the first one to become visible and is soon followed by the fourth pair. Subsequently, pairs five and one form approximately at the same time, and number two is the last one to emerge (Mailleux et al., 2002).

From E11 to E14 the proliferative activity of the mammary epithelium is very low compared to the adjacent epidermis. Similar results have been obtained in mice and rabbits (Balinsky,

1950; Lee et al., 2011). In addition previous studies using a scanning electron microscopy have revealed that in rabbits some of the cells residing atop the milk line have characteristics typical for motile, migrating cells such as philopodia (Propper, 1978). Indeed, the formation of placodes is thought to rely on migration of mammary precursor cells along and from the surrounding area of the milk lines. The influx of cells towards the future placode sites is thus the main source of mammary epithelial growth together with the increase of individual cell volume within the primordia (Kogata et al., 2014;Lee et al., 2011). However, only live imaging of the milk line during placode formation will offer definite proof of the matter.

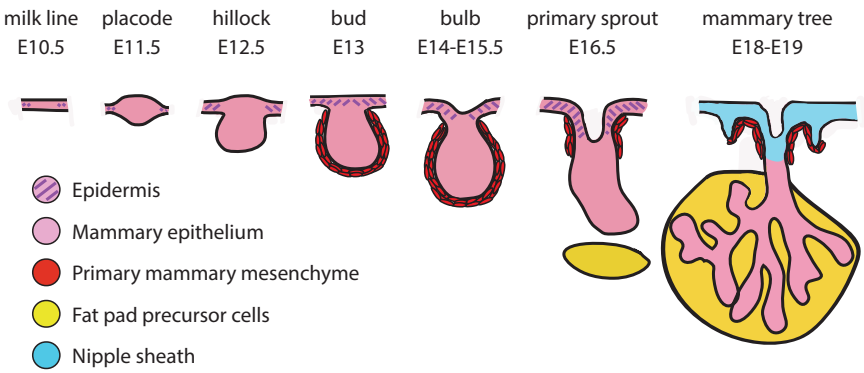

Figure 3. Embryonic mammary gland development.
The mammary rudiment development commences with the formation of milk lines at E10.5. By E11.5 all five pairs of mammary placodes have emerged. They gradually develop from hillock to a bud which is surrounded by the primary mammary mesenchyme. The bulb elongates to a primary sprout that grows towards the fat pad precursor cells. By birth, a small ductal tree with several branches has formed.

From bud to branching morphogenesis

The placode gradually transforms via hillock structure to a bud by E13 (Figure 3). By then, the mammary epithelium has grown downward and is surrounded by several layers of differentiated, condensed primary mammary mesenchyme. Further growth of the primordium gives rise to a bulb-like structure that is characterized by a narrow neck at E14 (Propper et al., 2013;Robinson et al., 1999). By E16.5 the tip of the primordium elongates to form a primary sprout, which invaginates through the dermis into the more distal secondary mammary mesenchyme or the fat pad precursor compartment. The fat pad precursor cells, together with the primary mammary mesenchyme, comprise the embryonic stroma that surrounds the mammary epithelium. Precursors of the fat pad, preadipocytes, are already present in the mouse embryo from E13.5 onwards but they only mature to adipose tissue couple of days after birth (Sakakura et al., 1982). The first branches begin to form from E16.5 onwards. The nipple sheath, thickening of the upper epithelium, which later on produces the nipple, appears at E17.5. Concurrently the ductal lumen formation commences with the appearance of small microlumens that progressively enlarge and join together (Hogg et al., 1983). By birth, approximately at E19, the lumen formation is complete, and a small ductal tree of 10-15 branches has developed.

The branching pattern of the mammary gland is stochastic. The precise number of ductal tips per time point is not predetermined and varies between mouse strains and even among

primordia of the same pair (Veltmaat 2003). The different mammary gland pairs also develop at individual speeds. By birth, the second and the third rudiments exhibit a more extensive ductal network compared to the other glands. This supports the notion that mammary glands are not identical and that they utilize at least partly divergent mechanisms for their individual development and growth (Propper et al., 2013;Veltmaat et al., 2003).

Significance of mammary mesenchyme and microenvironment

The importance of the mammary mesenchyme is apparent as epithelial mammary primordia fail to develop in its absence (Kratochwil, 1969). Indeed, the instructive signal for organ induction appears to originate from the mesenchyme. Tissue recombination studies have further demonstrated that the mammary mesenchyme can direct the morphogenesis of various ectodermal structures and regions towards a composition typical for mammary glands. This instructive capacity is highly conserved even across different species. Murine mammary mesenchyme is able to instruct the dorsal surface ectoderm of a rat embryo to form a mammary gland (Cunha et al., 1995).

Once mammary epithelium is recombined with mesenchyme originating from salivary gland and grown under a kidney capsule, the mammary-like branch pattern is lost, and the crafted organ resembles a salivary gland. Part of the mammary cell identity is retained, however, as the epithelium remains capable of producing proteins typical of milk composition in the correct hormonal environment. These experiments demonstrate that mesenchymal cues control the pattern of branching though the mammary cell fate remains unaltered (Sakakura et al., 1976).

In mice the initial development of mammary glands proceeds identically in both male and female embryos. By E14.5, the secretion of testosterone from the male testis activates androgen receptors (AR) that are present in the condensed primary mammary mesenchyme. This triggers apoptosis in the mammary mesenchyme as well as in the epithelium, and as a consequence the connection between the bulb and the surface epidermis is eventually severed (Heuberger et al., 1982;Kratochwil and Schwartz, 1976). The remaining mammary epithelium regresses and disappears. Even though males of some strains may retain an extremely rudimentary ductal system in adulthood, in most cases the mammary glands and nipples do not form in males (Dunbar et al., 1998;Durnberger and Kratochwil, 1980;Kratochwil and Schwartz, 1976;Kratochwil, 1977). However, in most other mammals, including human, the embryonic and prepubertal development are practically identical in both genders, and the sexual dimorphism of the glands does not occur until puberty.

1.4.2 Molecular regulation of development

From the milk line to the placode

The molecular regulation of the milk line specification and the placode induction has begun to receive more attention during the past decade. Substantial data have been gained by the study of different mouse models where downregulation or loss-of-function of a gene leads to impaired milk line formation and/or the absence of particular placode pairs (Table1.). Especially Fgf10, Nrg3, canonical Wnt signalling and transcription factors Gli3 and Tbx3 are necessary during the initial stages of mammogenesis (Cowin and Wysolmerski, 2010). Transcription factor p63 is also required for the commitment of the surface ectoderm to epidermal fate and induction of all ectodermal appendages, including mammary placodes (Mills et al., 1999).

Fgf10 is one of the earliest genes known to be essential for the specification of the milk line. *Fgf10* is expressed as a gradient from the ventral tips of the extended thoraric somites at E10.25. Its signalling partner, *Fgf2rb,* has been localized to the surface ectoderm and later to the emerging placodes (Mailleux et al., 2002;Veltmaat et al., 2006). In the absence of *Fgf10* or *Fgf2rb*, milk line formation becomes impaired and only placode four is able to develop (Mailleux et al., 2002;Veltmaat et al., 2006). The significance of somitic *Fgf10* in milk line positioning is further evidenced by the dorsalisation of the milk line in mutants where the ventral extension of the somites is undeveloped (Veltmaat et al., 2006). For mammary placodes one and five that are not located in close vicinity of the dermomyotomes, the signalling source of Fgf10 most likely is the adjacent limb bud mesenchyme (Veltmaat et al., 2006;Xu et al., 1998).

Expression of *Gli3* is found in the somites concurrently with *Fgf10*. Its actions appear to partly regulate *Fgf10* as loss of *Gli3* leads to downregulation of *Fgf10*. Attenuation of *Fgf10* has been proposed to be the reason behind the failure of placodes three and five to form in *Gli3* null and *Fgf10* hypomorph mice (Veltmaat et al., 2006). Positive Hedgehog signalling is necessary for the development of many ectodermal organs. In mammary glands, however, *Gli3* functions as a repressor of the Hedgehog pathway (Hatsell and Cowin, 2006). How the Gli3 repressor regulates *Fgf10* expression is not understood.

Tbx3 is first detected at E10.25 as a continuous streak in the mesenchyme of the presumptive milk line region. The expression shifts to the ectoderm prior to placode formation and finally accumulates in the emerging epithelial rudiments by E11.5. Mice deprived of *Tbx3* occasionally develop mammary placode number two, whereas other placodes are always lost (Davenport et al., 2003). Expression of a closely related member, *Tbx2*, is likewise found in the mammary mesenchyme at the onset of placode formation. However, the absence of *Tbx2* has no effect on mammary placode induction (Jerome-Majewska et al., 2005). In humans, haploinsufficiency of TBX3 results in ulnar-mammary syndrome which is characterized by severe mammary hypoplasia (Bamshad et al., 1997).

Tbx3 has been proposed to act both up- and downstream of Fgf and Wnt pathways (Chu et al., 2004;Eblaghie et al., 2004). During placode formation Tbx3 is required for the localized expression of *Wnt10b* and *Lef1* in the mammary epithelium and *in vitro* studies have further demonstrated that *Tbx3* potentiates Wnt and Fgf signalling (Davenport et al., 2003;Eblaghie et al., 2004). Experimental manipulations have also shown that on the ventral side of the embryo Tbx3 is antagonized by Bmp4. The opposing effects of the genes are believed to position the future milk line to the dorso-ventral border of the embryo by regulation and maintenance of positive Wnt activity (Cho et al., 2006). *Gli3* appears to control *Tbx3*/*Bmp4* patterning within the region of placode number three. In the absence of *Gli3*, *Bmp4* expression pattern broadens, which most likely in turn inhibits *Tbx3* (Chandramouli et al., 2013). Recent *in vitro* studies have also suggested that retinoic acid (RA) signalling induces or maintains *Tbx3* expression. Curiously, the receptor for RA is co-localized with *Fgf10* in the dermomyotomes of the somites, which might indicate a role in mammary placode induction and possible interactions with Fgf10/Fgfr2b signalling (Cho et al., 2012).

Another important molecule for embryonic mammary gland development is Nrg3 that is first observed prior to placode formation in the dermal mesenchyme. The expression switches to epithelial compartment as placodes mature (Wansbury et al., 2008). Placode pair three is especially sensitive to impaired Nrg3 signalling, as it is frequently absent or surrounded by ectopic placode-like structures in the hypomorphic Nrg3 mutants (*Scaramanga* mutants) (Howard et al., 2005). *Fgf10* and *Tbx3* expression remains unaltered in the *Scaramanga,* which

places *Nrg3* either downstream of the factors or indicates independent, parallel signalling. *Ex vivo* application of Nrg3 induces Wnt signalling and formation of placode-like structures that express *Lef1* (Howard et al., 2005). Gain-of-function K14-*Nrg3* mice on the other hand develop ectopic mammary glands, which further supports the role of *Nrg3* in mammary induction and promotion of mammary cell fate (Panchal et al., 2007).

The milk line was originally identified by low-level tripartite expression of *Wnt10b* at E10.5 (Veltmaat et al., 2004). As placodes emerge, the expression transiently connects all independent milk lines of a flank, whereas the placodes exhibit higher Wnt10b levels. Additionally, *Wnt6* and *Wnt3* are expressed prior to placode formation along the milk line in a broader pattern together with mesenchymal *Wnt5a* and *Wnt11* (Chu et al., 2004). *Lef1* is expressed at the onset of placode development, and as the placodes mature, it gradually becomes confined to the emerging epithelial structures and to the surrounding mesenchyme (Boras-Granic et al., 2006). Only three placode pairs develop in *Lef1*$^{-/-}$ embryos, and they eventually regress (van Genderen et al., 1994).

Overexpression of Wnt inhibitor *Dkk1* in the surface epithelium abolishes induction of all mammary primordia, which further underlines the necessity for proper Wnt signalling. *Dkk1* expression, however, does not alter *Fgf10* expression. This places Wnts downstream or independent of Gli3-Fgf10-Fgfr2b signalling (Chu et al., 2004). In line with this, TopGal and *Wnt10b* expression is not found in *Fgf10* hypomorph embryos or *Gli3* mutants (Veltmaat et al., 2006).

Ex vivo application of Wnt3a increases the placode size and artificial activation of the pathway with LiCl induces formation of ectopic placode-like structures along the flank (Chu 2004). *In vivo* loss of Sostdc1 function, a potent inhibitor of Wnt signalling, or its receptor Lrp4 causes upregulation of *β-cat* along the milk line, which most likely is the explanation for the ectopic nipple structures found in the adult females (Ahn et al., 2013;Närhi et al., 2012).

From bud to ductal tree

Transcription factors Msx1 and Msx2 are localized to the mammary epithelium and Msx2 additionally to the surrounding mesenchyme of the bud. Whereas the loss of only one factor has no drastic effect on bud formation, the disruption of both genes leads to unusually small placodes that do not develop beyond the bud stage (Satokata et al., 2000). In addition, Msx2$^{-/-}$ mice display a delayed branching morphogenesis that is, however, normalized soon after birth (Hens et al., 2007).

Pthrp is expressed from the placode stage onwards in the mammary epithelium, whereas its receptor, Pthr1, is located in the surrounding mesenchyme (Dunbar et al., 1998). The mammary primordia of *Pthrp*$^{-/-}$ embryos develop normally until the late bulb stage. However, the lack of Pthrp signalling disrupts the development of the primary mammary mesenchyme, and, as a result, initiation of ductal morphogenesis never occurs and the bulb reverts back to epidermal fate (Wysolmerski et al., 1998). *Pthrp* null embryos display severely reduced or completely abolished expressions of Tenascin-c, oestrogen receptor α (ERα) and AR in the primary mammary mesenchyme (Dunbar et al., 1999). Pthrp has also been shown to be responsible for mesenchymal Wnt activity and expression of *lef1* and *β-cat,* which are thought to mediate the specification of the mammary mesenchyme (Hiremath et al., 2012). The lack of mesenchymal AR in the *Pthrp* null embryos leads to a loss of sexual dimorphism as the androgen-induced destruction of the mammary bulbs does not take place (Dunbar et al., 1999).

During normal rudiment growth, Pthrp enhances the *Bmp1* receptor gene expression in the mesenchyme and thereby sensitises it to concurrently-expressed ligand *Bmp4*. Tissue culture experiments have demonstrated that application of Bmp4 can overcome the stunted bulb phenotype, indicating that it is an important downstream effector of Pthrp (Hens et al., 2007). Bmp signalling in turn has been shown to activate several factors important for further development (Hens et al., 2009).

In addition, *Pthrp* is important for nipple formation. The mammary mesenchyme instructs the keratinocytes of the proximal part of the mammary sprout to form a nipple sheath that later on develops into a nipple. Mesenchymal Msx2, which is induced by Pthrp, suppresses hair placode formation in the immediate surroundings of the future nipple. Expression of *Pthrp* throughout the basal layer of the skin under the K14-promoter redirects the entire ventral epithelium to adopt hairless nipple tissue fate (Foley et al., 2001;Hens et al., 2007).

The importance of Wnt signalling is not restricted to the early stages of mammogenesis (Incassati et al., 2010); rather it continues to play a role in the further development of the rudiments (Boras-Granic et al., 2006;Chu et al., 2004;Lindvall et al., 2006;Lindvall et al., 2009). The disruption of different pathway components leads to abnormal Wnt activity, which translates into impaired ductal development. *Lrp5* and *Lrp6* loss-of-function embryos exhibit decreased Wnt activity and abnormally small mammary placodes/buds. The subsequent outgrowth of the organ is delayed but otherwise normal in *Lrp5* mutants, whereas *Lrp6*$^{-/-}$embryos display a stunted branching morphogenesis and reduction in the size of the fat pad prior. Whether the observed phenotype of the mice is a result of epithelial or mesenchymal defects is not clear (Lindvall et al., 2006;Lindvall et al., 2009). Lack of Pygo2, a downstream component of Wnt signalling, leads to a similar phenotype with impaired mammary bulb growth and stagnant branching morphogenesis (Gu et al., 2009).

Fairly little is known about fat pad development, considering its immense importance for sustaining mammary gland formation and growth. Fat pad development requires Lef1, as the structures are unusually small in loss-of-function mutants (Boras-Granic et al., 2006). During late embryogenesis, expression of *Fgf10* has been localized to fat pad precursor cells, and the compartment is underdeveloped in *Fgf10*$^{-/-}$ embryos. The only epithelial rudiment pair that manages to form in the mutant embryos displays impaired branching morphogenesis. However, the ductal development can be rescued by transferring the rudiment to wild type stroma, which indicates that the observed phenotype is due to *Fgf10*-deficient fat pad (Mailleux et al., 2002). However, as Fgf10 signals via epithelial Fgfrs, the effect on the fat pad is likely indirect.

In addition to its early inductive role in mammogenesis, Gli3 is necessary for the later stages of development. Gli3 is required by primordia number two that fails to form a primary sprout or initiate branching morphogenesis and instead protrudes outward from the skin in *Gli3* null mutants (Chandramouli et al., 2013;Lee et al., 2011). The mice also fail to maintain proper mammary mesenchyme identity and exhibit downregulation of typical mesenchymal markers including AR. Scarcity of AR expression suppresses apoptotic destruction of the male mammary rudiment (Chandramouli et al., 2013).

Hoxc6 loss-of-function halts the ductal development of the thoracic primordia to the primary sprout stage and impairs the fat pad development (Garcia-Gasca and Spyropoulos, 2000). However, the signalling networks involved are unknown.

Finally, deficiency of the epidermal growth factor receptor (Egfr) diminishes branching morphogenesis but does not otherwise impair the embryonic sprouting or ductal development. Protease Adam17 is required to release Egfr ligand Areg from the cell surface. *Adam17* $^{-/-}$

neonates display an identical undeveloped ductal phenotype as *Egfr*$^{-/-}$ animals, which indicates that Areg is an important factor in the early ductal development (Sternlicht et al., 2005) (Table1.).

Table 1. *Selected mouse models that exhibit abnormal embryonic mammary gland phenotype. MP=mammary placode; MR=mammary rudiment.*

Mouse model	Phenotype	Reference
Altered placode induction		
p63-/-	No placodes	(Mills et al., 1999)
Fgf10-/-	Present: MP4	(Mailleux et al., 2002)
Fgfr2b-/-	Present: MP4	(Mailleux et al., 2002)
Gli3-/-	Present: MP1, 2, 4	(Hatsell and Cowin, 2006)
Tbx3-/-	Occasionally present: MP3	(Davenport et al., 2003)
K14-Dkk1	No placodes	(Chu et al., 2004)
Lef1 -/-	Present: MP1, 4, 5. Degenerate later.	(van Genderen et al., 1994)
Pygo2-/-	Small placodes/ buds	(Gu et al., 2009)
Lrp5-/- or Lrp6-/-	Small placodes/buds	(Lindvall et al., 2009)
Ska (Nrg3 hypomorph)	Occasionally ectopic MP around MP4	(Howard et al., 2005)
K14-Nrg3	Ectopic placodes	(Panchal et al., 2007)
Lrp4-/-	Ectopic placodes	(Ahn et al., 2013)
K14-Eda	Ectopic placodes	(Mustonen et al., 2003; Mustonen et al., 2004)
Altered morphogenesis after placode induction		
Msx2-/-	Arrest at sprout stage/delayed branching	(Satokata et al., 2000)
Msx1-/-;Msx2-/-	Stunted branching	(Satokata et al., 2000)
PTHrP-/- or Ptr1-/-	Arrest at bud stage	(Foley et al., 2001)
Gli3-/-	Stunted branching of MR2	(Chandramouli et al., 2013;Lee et al., 2011)
Fgf10-/-	Stunted branching of MR4	(Mailleux et al., 2002)
Areg-/-	Delayed branching	(Sternlicht et al., 2005)
Adam 17-/-	Delayed branching	(Sternlicht et al., 2005)

1.4.3 Postnatal development

Between birth and puberty the size of the mammary gland does not alter greatly. Growth of the organ is isometric, in other words it maintains but does not exceed the rate at which the animal increases body mass. Whereas the embryonic mammary gland has multiple layers of epithelial cells, the ducts of a postnatal gland are bilayered and contain two distinct cell populations; the outer basal or myoepithelial and inner luminal cells (Sternlicht et al., 2006). The embryonic

and prepubertal phases of the mammary gland morphogenesis is hormone-independent. The rudiments form normally in *oestrogen*, *prolactin*, *progesterone* and *growth hormone* receptor knock-out mice. However, all these factors are a necessity during the following, hormone-dependent, development (Bocchinfuso et al., 2000;Curtis Hewitt et al., 2000;Macias and Hinck, 2012).

At puberty, the morphology of the gland begins to change drastically as extensive ductal elongation and branching takes place. Bulbous, highly proliferative and multicellular terminal end bud (TEB) structures appear to the distal tips of the gland. Bifurcation of the TEBs creates new primary branches, whereas secondary branches sprout laterally from the trailing ducts. By week 12, the TEBs have reached the far ends of the fat pad and regress to terminal ducts. At this stage the elaborate ductal network fills a large portion of the fat pad. A recurrent oestrous cycle remodels the gland by producing transient tertiary branches off the secondary ducts (Sternlicht et al., 2006).

During pregnancy, the mammary gland undergoes further maturation to prepare for lactation. Widespread tertiary branching commences during early pregnancy and fills the majority of the empty space between the ducts. The tips of the newly-formed branches differentiate progressively into secretory alveolar cells that give rise to the milk-producing lobules of the lactating gland. Weaning initiates involution, another massive remodelling of the mammary epithelium. The obsolete milk-producing alveolar structures are removed from the gland either by apoptosis or by transdifferentiation to other cell types, and the gland reverts close to a state prior to pregnancy. The same cycle repeats with each pregnancy (Brisken and O'Malley, 2010;Sternlicht et al., 2006).

Morphogenesis after the onset of puberty depends on oestrogen, growth hormone, progesterone and prolactin. The mammary-gland-typical responses to hormonal signalling are mediated by specific hormone receptors located in the mammary epithelium and the stroma. Adolescent ductal development depends on oestrogen and growth hormone. The absence of oestrogen receptor alpha inhibits the formation of TEBs, which in turn impairs invasion of the epithelial ducts to the fat pad (Mallepell et al., 2006). The lack of growth hormone receptor induces highly similar defects in adolescent mice (Gallego et al., 2001). Pregnancy-induced side branching depends instead on progesterone, as the structures do not form in females with deficient progesterone receptors. Progesterone also controls alveologenesis together with prolactin (Brisken et al., 1998;Ormandy et al., 1997). The systemic hormonal cues are eventually translated to local paracrine messages that mediate their effects with distinct factors. The full scale of hormone interaction with developmental signalling pathways in mammary gland branching morphogenesis and differentiation is still being discovered.

Of the developmental pathways that are employed in embryonic mammogenesis, many are also active during hormone-dependent stages. Areg is an essential mediator of oestrogen-induced proliferation during puberty (Ciarloni et al., 2007), whereas growth hormone is believed to act mainly through Insulin-like growth factor 1 (Igf1) (Kleinberg et al., 2000). Furthermore, Fgfr2b is required for TEB maintenance (Lu et al., 2008;Parsa et al., 2008) and Fgf10 has been shown to regulate branch initiation (Zhang et al., 2014). Though loss of *Wnt5a* does not alter embryonic mammary gland induction (Chu et al., 2004), it accelerates and enhances ductal growth during hormonal development by increasing lateral branching and TEB size (Roarty and Serra, 2007). R-spondin1 was recently shown to mediate hormone action and to be upregulated during different stages of pregnancy (Cai et al., 2014). In addition, *Wnt4* and Tnf family member *RANKL* and have been identified as a progesterone target genes (Beleut et al., 2010;Brisken et al.,

2000;Mulac-Jericevic et al., 2003). NF-κB on the other hand has been recognized as a positive regulator of ductal growth during adolescence and pregnancy (Brantley et al., 2000;Cao and Karin, 2003).

1.5 Variations in mammary gland number

The number and location of the mammary glands present in adult animals vary widely between different mammalian species. Humans and other primates have one thoracic pair, whereas ungulates such as goats, sheep and horses have their only pair located in the inguinal part of the ventral body. Similarly, whales have only one mammary gland pair which is located near the genitalia and manatees have one nipple in each armpit (Rodrigues et al., 2014). Several animals also exhibit multiple pairs of mammary glands. Domesticated pigs for instance can have as many as nine sets of glands that run from the axilla towards the groin. The teat number in pigs is a variable trait and is commonly used as an indicator of reproductive performance (Xu et al., 2014). Though the regular house mouse (*Mus musculus*) has its five mammary gland pairs distributed in both the upper torso and groin region, other rodent species display much more variation in the number of glands (Gilbert, 1986). The most extreme case is the multimammate mouse (African soft furred rat) of *Mastomus* genus that can have up to 12 equally-spaced nipple sets located throughout the ventral region from axilla to groin (Brambell et al., 1941).

The number of glands correlates closely with the amount of offspring typically birthed at a time. Most commonly the litter size is one half of the gland number and rarely exceeds their amount (Bresslau, 1920;Diamond, 1987;Gilbert, 1986;Sherman et al., 1999). With only a few exceptions, the organs are located symmetrically along the anterior-posterior axis, and they form in conserved positions within a species (Koyama et al., 2013;Sherman et al., 1999).

1.5.1 Supernumerary mammary glands in humans

In humans, supernumerary nipples or polythelia are fairly typical developmental abnormalities with prevalence ranging from 0.2% to 5.6% (Ferrara et al., 2009). The number of the accessory nipples is not constant and can vary from one to even seven (Goyal et al., 2012). These structures are occasionally associated with breast tissue (polymastia) and have been described frequently over the past few centuries in both genders (Kajava, 1915;Loukas et al., 2007;Velanovich, 1995;Williams, 1891). Often additional nipples and associated glandular tissue are very rudimentary compared to endogenous breasts though they appear to respond to hormonal stimulation and undergo physiological changes similarly. Ectopic nipples can for instance secrete milk, and the glands are often reported to increase in size during pregnancy (Williams, 1891).

Accessory nipples generally form within the area defined by the embryonic milk lines which extend from the armpit to the pubic region. On occasion, ectopic nipples have also been described in unusual locations, such as the face and the sole of the foot (Balakrishnan and Madaree, 2010;Conde et al., 2006). Most often polythelia arises as a sporadic phenomenon. However, it can also be inherited as a solitary trait or as familial aggregation, where polythelia is one of several other defects that manifest within a family. Kidney and urinary tract abnormalities are the most frequent associations with supernumerary nipples (Ferrara et al., 2009). Genetic disorders such as Simpson–Golabi–Behmel syndrome have occasionally been described with supernumerary nipples (Neri et al., 1998).

Ectopic nipples are not restricted to humans but are also reported in other primates (Hsu et al., 2000). Supernumerary teats are likewise a frequent abnormality of the bovine udder (Gifford, 1934;Pausch et al., 2012).

1.6 Relevance of embryonic mammary gland development to breast cancer

Breast cancer is a highly heterogenous disease that can arise in various parts of the mammary gland such as epithelial ducts, milk producing lobules and the mesenchymal stroma. On average every 8th woman is affected by breast cancer during her lifetime, although factors such as ethnic origin, lifestyle, environment, family and reproductive history can have an impact on the occurrence. Breast cancer is not restricted to women but also affects men, though the number of incidences is fairly small (http://www.cancer.org/cancer/breastcancer). Similarly, ectopic breast tissue that is occasionally associated with supernumerary nipples is susceptible to oncogenesis with low frequency (Francone et al., 2013).

The manner in which the embryonic mammary glands form manifests parallel characteristics to certain features typical to breast cancer (Howard and Ashworth, 2006). During embryogenesis, processes that are required for mammary gland development, such as cell migration, proliferation, differentiation and ductal invasion to the surrounding mesenchyme, occur in a tightly controlled and coordinated manner. Cancerous cells utilize similar mechanisms for growth and metastasis, invasion of the cancer cells to other parts of the body. Many of the signalling pathways that are needed for ordinary mammogenesis become misregulated in breast cancer. Transcription factor Tbx3 has for example been associated with breast cancer (Douglas and Papaioannou, 2013). Similarly, breast cancer cells have often been found to possess constitutively activated NF-κB (Shostak and Chariot, 2011;Sovak et al., 1997). ErbB4, receptor for Nrg3, on the other hand can heterodimerize with ErbB2, which has a profound link to breast cancer (Holbro et al., 2003). Several studies have also implicated Pthrp and Wnts in breast cancer and metastasis (Boras-Granic and Wysolmerski, 2012;Howard and Ashworth, 2006).

Induction and growth of the epithelial mammary rudiment relies on mesenchymal signalling. During postnatal life, the mammary stroma supports and maintains the development of the gland. Tissue recombination experiments have shown that the combination of epithelial mammary tissue with the embryonic primary mammary mesenchyme results in a hyperbranched ductal phenotype, whereas growth with the fat pad gives rise to a gland that has undergone regular branching morphogenesis (Sakakura et al., 1987). The tumour microenvironment might resemble the embryonic mammary mesenchyme in this regard, and exposure of the epithelial structures to such conditions might promote initiation and progression of breast cancer (Sakakura et al., 2013). Research on tumour microenvironment has become a topic of greater interest in recent years.

Thus, understanding the normal developmental processes and how different signalling pathways contribute to morphological changes and modulate each other's function may help to understand the processes that are responsible for breast cancer. Targeted modulation of certain signalling pathways or utilizing their innate feedback mechanisms has indeed been exploited in cancer therapies and drug discovery (Curtin and Lorenzi, 2010;Howard and Lu, 2014).

2 Aims of the study

The absence of Eda impairs the morphogenesis of many epithelial appendages, but prior to this work, relatively little was known about Eda's function in mammary gland development. Previous reports had indicated that an excess of Eda stimulates ectodermal organ development. Strikingly, overexpression of Eda had been shown to induce formation of supernumerary mammary placodes within the milk line (Mustonen et al., 2003;Mustonen et al., 2004) suggesting the involvement of Eda also in mammogenesis. Most Eda downstream effectors had been identified in organs such as teeth and hair, yet the downstream pathways and target genes of Eda in mammary glands were unknown.

The aims of the thesis are as follows:

1) To characterize Eda gain- and loss-of-function mammary primordia phenotypes during several stages of prepubertal mammary gland morphogenesis.

2) To study the relevance of transcription factor NF-κB in mammogenesis during prepubertal development and to assess its possible significance as a downstream mediator of Eda.

3) To identify and validate potent Eda downstream targets in mammary primordia.

4) To develop *ex vivo* culture methods for studying embryonic mammary gland development, which can be utilized to evaluate the effects of individual Eda downstream effectors on placode formation and branching morphogenesis.

3 Materials and methods

In my thesis work, the mouse was used as a model organism. *TopGal* and *BatGal* reporter mice were used to determine activity of Wnt signalling and NF-κB reporter strain to observe NF-κB activity. *Eda* gain- and loss-of-function mice (K14-*Eda* and *Eda* $^{-/-}$) were utilised to elucidate the role of Eda in mammary gland development and *IκB*αΔN mouse line to study importance of transcription factor NF-κB. The mice strains were maintained in *NMRI*, *C57BL/6*, *FVB* or *B6CBA* background.

Mouse strain	Reference	Used in article
NMRI	Jackson Laboratories	I, II, III
C57BL/6	Harlan	I, II, III
B6CBA	Harlan	III
FVB	Harlan	III
TopGal	(DasGupta and Fuchs, 1999)	II, III
BatGal	(Maretto et al., 2003)	III
NF-κB gal	(Bhakar et al., 2002)	II,III
K14-Eda	(Mustonen et al., 2003)	I, II, III
Eda$^{-/-}$ *(Tabby)*	Harlan	II, III
*IκB*αΔN	(Schmidt-Ullrich et al., 2001)	II, III

Methods that were used in this thesis work are described in detail in the original articles and the manuscript. Tissue culture of embryonic mammary rudiments was an essential part of observing branching morphogenesis and placode formation and is discussed more thoroughly in the results and discussion section.

Method	Used in article
Immunological detection of proteins	
Immunohistochemistry	II, III
Immunofluorescence	III
Gene expression analysis	
Whole-mount in situ hybridization	I, II, III
Non-radioactive in situ hybridisation on sections	II, III
Radioactive in situ hybridisation on sections	II, III
Quantitative RT-PCR	II, III
Affymetrix Mouse Exon 1.0 ST microarray	II
Microscopy	
Bright field microscopy	I, II, III
Fluorescent microscopy	II, III
Confocal microscopy	III
Scanning electron microscopy	II
Tissue culture	I, II, III
mammary rudiment branching morphogenesis	I, III
mammary placode formation	II
Others	
Histology	II, III
Carmine alum staining of mammary glands	I, II, III
X-gal staining	II, III
Cell proliferation assay (EdU)	III

Analysis of gene expression pattern by radioactive in situ hybridization was done by using ^{35}S-UTP labelled probes and non-radioactive in situ hybridization was done by using digoxigenin labelled probes. In the following table the probes are listed and their original references are indicated.

Probes	**Reference**	**Used in article**
Lgr4	article II	II
Kremen2	article II	II
Dkk4	(Fliniaux et al., 2008)	II
Lef1	(Travis et al., 1991)	II
Wnt10a	(Dassule and McMahon, 1998)	II
Wnt10b	(Wang and Shackleford, 1996)	II, III
β-*cat*	(Laurikkala et al., 2002)	II
Shh	(Vaahtokari et al., 1996)	II
Pthrp	article III	II, III
Nrg3	(Howard et al., 2005)	II
Tbx3	(Howard et al., 2005)	II
Mmp9	article II	II
Adamts-15	article II	II
Edar	(Laurikkala et al., 2001)	II, III
Eda	(Laurikkala et al., 2001)	II, III
Areg	article III	III
Epgn	article III	III

4 Results and discussion

4.1 Ex vivo culture of mammary primordia (I, II, III)

Embryonic explant cultures sustain tissue and its normal interactions in a closed, artificial system that allows the monitoring and precise manipulation of morphogenesis. To date, embryonic mammary glands have not been studied as thoroughly as some ectodermal organs, such as hair, teeth and salivary glands, or other branched organs such as lungs and kidneys. As a result, very few articles have described techniques for culturing the embryonic mammary glands. Instead, much of the research has focused on adolescent or pregnancy-induced epithelial morphogenesis. For example, the mammosphere assays and the transplantation of epithelial cells to cleared fat pads are frequently used, well-established methods for assessing stem cell/progenitor cell activities and hormone-dependent morphogenesis (Brill et al., 2008;Shaw et al., 2012). Three-dimensional mammary organoid cultures consisting of isolated pieces of epithelial ducts, have been adopted to assess and image various aspects of mammary epithelial cell behaviours including branching morphogenesis (Ewald, 2013;Shamir and Ewald, 2014), but these culture systems lack the normal epithelial-mesenchymal tissue interactions known to be critical for mammary gland development. In order to study mammary placode induction and branching morphogenesis we developed two *ex vivo* culture systems.

Previously, formation of mammary placodes has been assessed by culturing E10.5 to E11.5 stage embryos that have been sagitally dissected into two symmetric halves (Chu et al., 2004;Kogata et al., 2013). Accordingly, I cultured E10.5 to E11.5 explants in a Trowell-type setup, a well-established technique, where tissues are kept at the gas-medium interface on top of a thin membrane that is supported by a metal grid (Närhi and Thesleff, 2010;Sahlberg et al., 2002;Trowell, 1959). I utilised control and K14-Eda embryos expressing the K17-GFP transgene (Bianchi et al., 2005) that marks the forming mammary placodes and monitored the development for 2-3 days. Although the endogenous primordia emerged under these culture conditions within two days in both genotypes, the ectopic placodes of K14-*Eda* embryos were not able to develop (Figure 4).

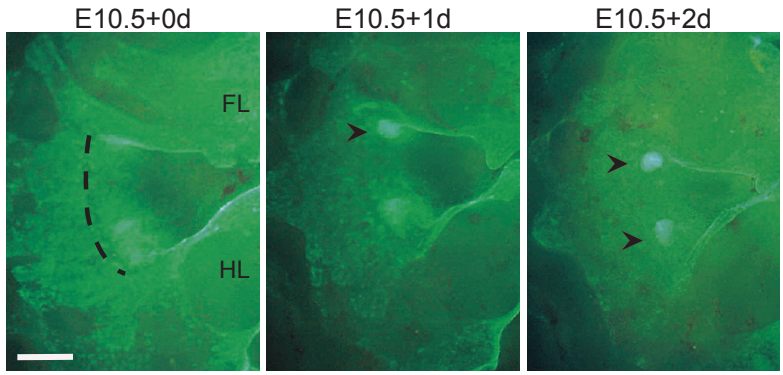

Figure 4. K17-GFP half embryo cultures.
K17-GFP half embryos were cultured from E10.5 onwards. During the culture period mammary placodes 3 and 4 emerged on the flank. However culture of K14-Eda embryos did not yield formation of the supernumerary placodes (not shown). Dashed line marks the milk line region, FL=fore limb, HL=hind limb, arrowheads point to the emerging mammary primordia. (Scale bar: 500 μm*)*

Overexpression of Eda is evident in the ectoderm already at E10.5 onwards (Mustonen et al., 2003), yet the supernumerary mammary placodes do not form until E12.5 to E13. The delayed development of the ectopic mammary placodes is the likely explanation to why I could not observe them in the explant cultures initiated already at E10.5 or E11.5. For this reason, I next cultured E12.5 flank skins, which comprised epithelial and mesenchymal compartments of the mammary forming region. In the explants, the endogenous mammary placodes had already formed, whereas the rest of the milk line region remained undeveloped. After two days of culture (E12.5+2d) the K14-*Eda* explants had developed placode-like structures between the third and the fourth mammary bud whereas the wild type explants displayed no such composition. Commonly, formation of two to three supernumerary primordia were observed, which corresponded to the adult phenotype of K14-*Eda* mice. Application of Fc-Eda uncovered the same mammary forming potential of the milk line. When wild type flank skins were cultured for two days (E12.5+2d) in the presence of the recombinant protein, mammary placode-like structures appeared in the flank. These results indicate that the application of Fc-Eda to wild type skins recapitulates the K14-*Eda* phenotype (Figure 5).

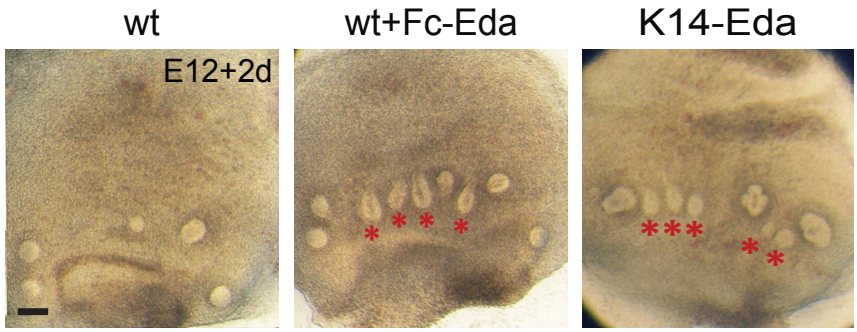

Figure 5. Ex vivo culture of E12.5 mammary forming region.
In wild type explants the five endogenous mammary buds are present. Supernumerary mammary placodes (*) form in K14-Eda explants between the 3rd and the 4th and the 2nd and the 3rd endogenous mammary rudiment. Application of recombinant Eda protein to wild type explants induces a phenotype mimicking K14-Eda. (Scale bar: 100 μm*)*

Ex vivo culture for observing later stages of mammary morphogenesis was first described in the late 1960s by Klaus Kratochwil (Kratochwil, 1969). His work revealed that the mammary rudiments are unable to sustain or induce their development in the absence of the underlying mesenchyme. He showed that the embryonic rudiments from several stages ranging from E11.5 to E15.5 could be maintained in culture conditions for up to nine days. The method relied on isolation of the mammary epithelium from the epidermis and mechanical scraping of the ventral mesenchyme that was used as a substrate to support epithelial morphogenesis (Kratochwil, 1969). A highly similar approach was utilized by Hens and colleagues for studying downstream effects of Pthrp (Hens et al., 2009;Hens et al., 2007). Whereas the described methods were obviously functional, we found them to be time consuming and the rate of successful development to be inconstant. The particular challenge of the procedure was the manual gathering of the ventral mesenchyme, which easily damaged the sensitive embryonic tissue. However, previous studies

have reported that by applying appropriate enzymatic processing it is possible to separate the embryonic epithelial and mesenchymal compartments without causing irreversible tissue damage (Drews and Drews, 1977).

In order to analyse factors affecting embryonic mammary gland branching more carefully, we developed a novel *ex vivo* mammary bud culture method. We modified the procedures described above by combining them with enzymatic separation of the epithelium and mesenchyme. We used pancreatin-trypsin as the proteolytic enzyme, but also dispase could be optimized for this purpose. We chose to use E13 stage explants for two particular reasons. First, at this time the androgen-mediated destruction of the male mammary rudiment has not yet initiated, which enables the use of all embryos regardless of their gender. Second, at E13 the branching morphogenesis has not commenced, which permits manipulation and detection of the ductal development from early stages.

To harvest an explant from the embryo, I dissected ventral flanks that contained both epithelial and mesenchymal compartments of the mammary forming region, and most often mammary buds two, three and four. The explants were treated with pancreatin-trypsin and the enzymatic activity was terminated by 10% FCS. After a short recovery from the proteolytic processing, the epidermis was peeled off from the mesenchyme. Despite the separation of the epidermis, the epithelial mammary buds typically stayed attached to the mesenchyme. This gave rise to an explant that comprised the ventral flank mesenchyme together with epithelial mammary buds at their original locations. At E13, the epidermis is still transparent and mammary rudiments are readily detected from whole mount embryos. During the following days, the epidermis keratinizes and thickens. Were it not removed prior to the culture period, it would eventually hinder the detection and visualization of the ductal development.

At E13, mammary buds are morphologically fairly quiescent and branching morphogenesis does not commence until at E16-E17 (Cowin and Wysolmerski, 2010;Veltmaat et al., 2003). Accordingly, during the first three days of the culture (E13+3d) the mammary buds merely grew in size but did not exhibit any other obvious morphological changes. On the fourth day, the mammary bud had adopted a more elongated structure, which would indeed correspond to the E16 stage and formation of the primary sprout. By the fifth day (E13+5d), the mammary rudiments showed more advanced ductal development and most often several distinct ductal tips. With wild type explants, the method yielded success rates from 70% to 90% depending on the mouse strain.

4.2 Eda promotes mammary placode formation and enhances prepubertal branching morphogenesis (II, III)

Eda is dispensable in the earliest stages of mammogenesis; *Eda* null embryos develop all five pairs of mammary placodes in conserved positions (Pispa et al., 2008). Transgenic overexpression of *Eda* (K14-*Eda* mice) gives rise to supernumerary mammary placodes along the milk line (Figure 6) that develop into small ductal trees and are associated with nipples in the adult animal. To better understand the effects of Eda on hormone-independent mammary gland development, we analysed both *Eda* gain- and loss-of-function mammary primordia at several prepubertal stages. We utilized whole mount *in situ* hybridization (WMISH) for several placode markers in addition to morphological analysis to visualize placode formation.

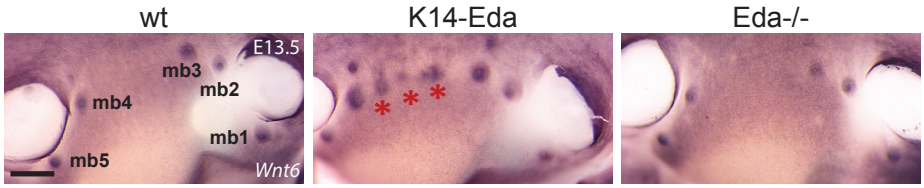

Figure 6. Whole mount in situ hybridization for Wnt6.
Wnt6 is expressed in the endogenous mammary buds of wild type, K14-Eda and Eda $^{-/-}$ embryos. By E13.5, supernumerary mammary placodes (*) have emerged in the mammary-forming region in K14-Eda background and likewise express Wnt6. (Scale bar: 500 μm*)*

Induction of the endogenous mammary placodes between E11 to E11.5 did not present noticeable defects between *Eda* $^{-/-}$ and wild type. Likewise, the endogenous mammary placodes in K14-*Eda* embryos developed seemingly normally and in appropriate positions.

Characterization of the supernumerary mammary placodes in K14-*Eda* mice revealed that their development was delayed compared to the endogenous rudiments as previously reported (Mustonen et al., 2004). By E13 stage the expression of several placode markers became restricted to the accessory structures. Traditionally the milk line that possesses the capability to generate mammary primordia has been considered to cover the regions between the fore and hind limbs (Veltmaat et al., 2004). Surprisingly, we found that K14-*Eda* mice develop ectopic placodes in the neck, an area previously not known to possess mammary gland potential. Unlike the supernumerary structures of the flank, the placode pair in the collar section initiated its development nearly simultaneously with endogenous placodes. Curiously, our *in situ* hybridization analysis of E11.5 wild type and K14-*Eda* embryos revealed a cluster of *Dkk4* and *Wnt10b* positive cells in the neck region of both genotypes. In wild type embryos the focal expressions were only transient, but in K14-*Eda* embryos they were sustained. These results could indicate that a mammary pre-placode forms regularly in the neck of wild type embryos. However, only elevated Eda levels appear to stabilize their further growth. Taken together, this suggests that the potential milk line in fact extends further that previously described.

Mutations of the Eda signalling pathway lead to a wide spectrum of characteristics from completely abolished development to milder alterations in organ formation (Mikkola, 2009). Eda has also been shown to fine-tune the development of several ectodermal organs (Sadier et al., 2014). Upregulation of the signalling output often increases placode size and number and alters the shape of the future organ (Häärä et al., 2011;Harjunmaa et al., 2012;Mustonen et al., 2004;Sadier et al., 2014). Our results show that upregulation of Eda has very similar effects on mammary placode formation. Early *Eda* $^{-/-}$ and K14-*Eda* phenotypes suggest that mammary induction is independent of the pathway. Instead, modulation of the Eda levels is more likely able to boost the existing potential residing along the entire milk line.

Quantitative analysis of E14.5 K14-*Eda* mammary bulbs confirmed a prominent increase in epithelial volume and cell proliferation. High proliferation coincided with precocious branch formation in the ductal epithelium in K14-*Eda* background. At E15.5 the wild type and *Eda* null rudiments were still at a bulb stage, as expected (Propper et al., 2013), whereas K14-*Eda* primordia had already elongated and the formation of the first branches was evident in the most advanced pairs. By E16.5, the transgenic ducts had developed into a small ductal tree that resembled new-born wild type glands.

At the onset of branching morphogenesis, between E15 to E17, expression of *Edar* was detected in the mammary epithelium, whereas *Eda* transcripts were localised to the surrounding mesenchyme. Classic tissue recombination studies have shown that the mammary rudiment is unable to develop in the absence of the mammary mesenchyme, which suggests that the branching pattern is determined by the mesenchyme (Dunbar et al., 1998;Kratochwil, 1969;Sakakura et al., 1976). Yet the precise role of many mesenchymal signals remains poorly understood, and most likely several are still waiting to be discovered. Few of the studied mesenchymal factors include somitic Fgf10 and Nrg3 that are found in the mesenchyme prior to placode induction (Howard et al., 2005;Mailleux et al., 2002). Later, *Fgf10* is present in the fat pad precursor cells. Additionally, *Fgf7* is expressed first in the primary mammary mesenchyme and is later co-expressed with *Fgf10* in the future fat pad compartment (Mailleux et al., 2002). Our results indicate that Eda is a novel mesenchymal factor that regulates mammary morphogenesis in a paracrine manner through activation of the epithelial Edar receptor.

Examination of the prenatal mammary glands at E18 revealed significant differences in epithelial ductal morphogenesis between all the genotypes. *Eda* loss-of-function led to a reduction in the amount of branches (68%), whereas a dramatic five-fold increase was observed in K14-*Eda* glands. Analysis of prepubertal 3-week-old mice confirmed that the detected phenotypes were not transient (Figure 7). K14-*Eda* animals displayed a three-fold increase in the number of ductal tips as compared to wild type controls, and in contrast an overall 54% reduction in the number of ductal termini was observed in *Eda* $^{-/-}$ animals. Taken together, this shows that embryonic and prepubertal branching correlates with the amount of Eda.

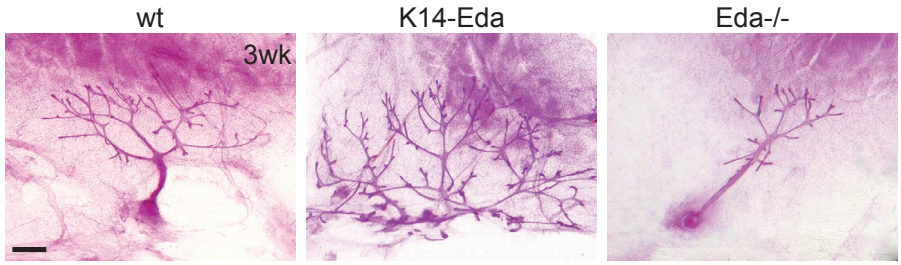

Figure 7. Branching morphogenesis in 3-week-old mice.
The branching morphogenesis correlates with the amount of Eda. K14-Eda exhibit increased amount of ductal tips whereas Eda$^{-/-}$ have fewer branches compared to wild type (Carmine alum stainings of mammary gland number 4). (Scale bar: 1 mm)

Despite the prominent increase in the size of the K14-*Eda* ductal tree at E18, the glands displayed a relatively typical mammary gland branch pattern. By birth, mammary glands two and three are always characterized by a more advanced ductal morphology than the rest of the rudiments (Veltmaat et al., 2003). The same asynchronous development was conserved in K14-*Eda* glands. This suggests that the observed increase in epithelial proliferation accelerating the branching morphogenesis is the main means for changes in ductal development. Similarly, analysis of *Eda* gain- and loss-of-function submandibular salivary gland has previously shown that Eda levels affect the branching morphogenesis of the organ but preserve the branch pattern typical to salivary glands (Häärä et al., 2011).

The *ex vivo* culture recapitulated the branch pattern of different mouse models. K14-*Eda* mammary rudiments showed a precocious and clearly more advanced branch phenotype after five days of culture compared to the wild type (Figure 8). However, *Eda* $^{-/-}$ mammary buds were most often unable to sustain their development *ex vivo,* indicating that the culture environment was somewhat harsher than *in vivo* conditions. Application of Fc-Eda however increased the percentage of successful ductal outgrowth from 10% to 50%.

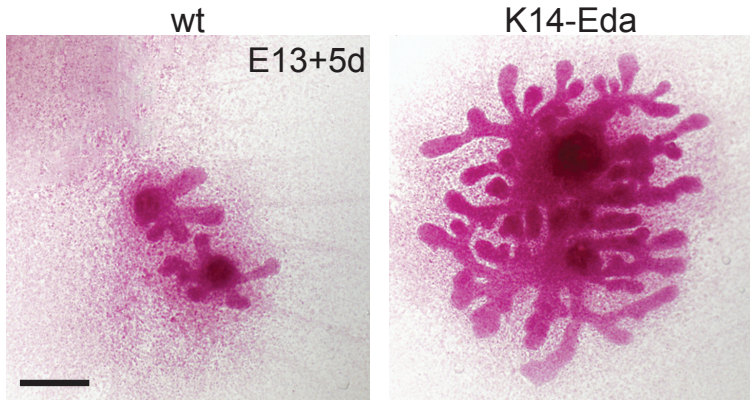

Figure 8. Ex vivo culture of E13.5 mammary buds.
After five days of culture the mammary buds have undergone branching morphogenesis that is significantly more advanced in K14-Eda explants. The mammary rudiments, thus, recapitulate the in vivo mammary phenotypes of the strains. (Carmine alum stain of mammary rudiments 2 and 3). (Scale bar: 250 μm*)*

The precise time window for Eda's function remains as of yet undetermined. Our analysis of *Eda* gain-of-function mammary glands has been conducted in a mouse model in which *Eda* is overexpressed as early as E9.5 and onwards. The activation of the transgene prior to mammary placode formation might, thus, affect the number of mammary progenitor cells, which in turn could give rise to the observed ductal phenotype. However, the ability of recombinant Eda protein (Fc-Eda-A1) to rescue ductal outgrowth of *Eda* $^{-/-}$ mammary buds *ex vivo* points towards a regulatory function during ductal morphogenesis. Creation and investigation of inducible gain- or loss-of-function mouse models could clarify the issue in the future. Alternatively, *ex vivo* application of Fc-Eda, agonistic anti-Edar, or function-blocking anti-Eda antibodies (Kowalczyk-Quintas et al., 2014;Kowalczyk-Quintas and Schneider, 2014) at different time points could be utilized to pinpoint the exact role of Eda at various developmental stages.

A recently-generated mouse knock-in model of the human *EDAR* gain-of-function variant (V370A) has been shown to exhibit an increase in the mammary branch density during adolescence (Kamberov et al., 2013). The mouse recapitulates many features associated to the variant in human populations (Kamberov et al., 2013). Similarly, transgenic mice with enhanced Edar signalling (*Edar*^{TG951}) as a result of a high copy number of the wild type *Edar* locus, show escalated ductal morphogenesis in adulthood. The same study likewise reported decreased branching in response to loss of *Eda* or *Edar* (Chang et al., 2009). Though these reports did not focus on prepubertal development, they suggest that ductal branching correlates with Eda levels and the effects might be similar in humans. Since the Eda pathway is often dispensable

for initiation of glandular organs (Gruneberg, 1971), including mammary glands, and instead necessary for epithelial growth and branching, it might be utilized in adaptive evolution to elevate the function of exocrine glands by increasing epithelial secretory area.

Breast defects associated with HED have not been extensively studied in human patients. Mammary glands mature to their functional form only upon pregnancy, and the identification of possible morphological defects that result in impaired lactation is hence bound to the individual's reproductive history. For this reason, the defects may remain latent for decades or never surface. Shortcomings in salivary and sweat gland function have more profound impact on the quality of everyday life, whereas aesthetic effects of sparse hair and malformed teeth are more self-evident. Thus far only one study has reported difficulties related to lactation in XLHED carriers. Insufficiency in milk production was extremely common (Clarke et al., 1987). However, these results were not compared to a control group of women. An unpublished study by National Foundation for Ectodermal Dysplasia (NFED) revealed that HED has profound and thus far unrecognized effects on breast development and lactation. According to the study, 35% of female HED patients have absent or hypoplastic nipples and only one third are able to breastfeed. This, together with individual case reports on mammary hypoplasia in association with HED, suggests that breast abnormalities are a frequent manifestation of the syndrome (Haghighi et al., 2013;Megarbane et al., 2008;NFED, 2012), but perhaps was previously neglected as the majority of the individual affected by HED are males.

Our results showing defects in prepubertal *Eda* $^{-/-}$ branching morphogenesis, and the report with a similar phenotype in post pubertal glands (Chang et al., 2009) suggest that a reduction of epithelial breast tissue could account for the described symptoms in humans. Role of Eda during pregnancy-induced tertiary branching has not been studied, and thus it cannot be excluded that impaired alveologenesis could bring about difficulties in breastfeeding. Normally *Eda* $^{-/-}$ mice are capable of feeding their young indicating that pregnancy-induced mammary gland maturation must be intact at least to some extent. However, a recently described rat model for HED, which harbours a loss-of-function mutation in the *Edaradd*, presents impaired alveolar growth and hypoplastic mammary glands (Kuramoto et al., 2011), which might indicate a possible role for Eda in pregnancy-associated ductal development.

4.3 NF-κB mediates effects of Eda during mammary primordia development (II, III)

Transcription factor NF-κB is believed to be the main downstream effector of Eda in many ectodermal organs (Mikkola, 2009). This conclusion is further strengthened by phenotypic similarities between *Eda* $^{-/-}$ and a mouse model with supressed NF-κB activity (*IκB*αΔN mice) (Schmidt-Ullrich et al., 2001). However, involvement of the JNK pathway has also been suggested by *in vitro* studies (Kumar et al., 2001), and other downstream mediators commonly activated by other Tnfrs cannot be excluded. The necessity of NF-κB in embryonic mammary gland development has thus far remained largely unexplored. According to a previous report, mammary epithelium displays high, Eda and Traf6 -dependent, NF-κB activity (Dickson et al., 2004;Pispa et al., 2008). We analysed NF-κB reporter expression in control and K14-*Eda* littermates to elucidate the spatiotemporal control of NF-κB activity and characterized how the involvement of Eda contributes to it.

At E11, the NF-κB activity was localized to the emerging mammary placodes and was fairly similar between the wild type and K14-*Eda* embryos. By E11.5, both genotypes exhibited a continuous stripe of low-level reporter-positive cells which combined the entire milk line region and was upregulated in the mammary primordia. From this stage onwards, NF-κB activity appeared to become downregulated in the intra-placodal region of wild type embryos, whereas more intense reporter activity was evident in the corresponding region in the K14-*Eda* embryos. By E13, low-level NF-κB activity had confined to supernumerary placodes in K14-*Eda* embryos. The ectopic placodes of the neck and the supernumerary placodes between the third and the fourth mammary bud exhibited a mosaic reporter expression. We also observed that the magnitude of NF-κB activity was dependent on Eda levels; $Eda^{-/-}$ mammary rudiments displayed no NF-κB activity, in wild type mice the reporter expression became confined to the basal cells from the bud stage onwards, and in K14-*Eda* embryos the entire mammary epithelium was characterized by ectopic reporter expression at multiple developmental stages.

In order to further understand the effects of NF-κB in mammary development, we utilized the *IκB*αΔN mouse line. The strain expresses non-degradable super-repressor IκBα ubiquitously, which leads the suppression of NF-κB activity (Schmidt-Ullrich et al., 2001). Similarly to $Eda^{-/-}$ mice, *IκB*αΔN animals retain all five pairs of mammary glands, and placodes form at conserved positions. At E18, *IκB*αΔN mammary glands exhibited a 48% decrease in ductal tip number compared to wild type littermates, and approximately the same difference was maintained at 3weeks of age. This indicated that NF-κB regulates normal embryonic ductal development, though it is not necessary for placode induction.

Compound K14-*Eda* and *IκB*αΔN embryos, however, revealed that the formation of supernumerary mammary primordia was dependent on NF-κB. The accessory structures and localized gene expression of all studied placode markers were absent in K14-*Eda*/*IκB*αΔN mutants. Additionally, we found that the hyperbranched K14-*Eda* ductal phenotype was normalized in the *IκB*αΔN background in E18 and 3-week-old mice. This clearly shows that NF-κB is a major transducer of Eda-induced placode formation and ductal branching. However, based on our results, the possible existence of other downstream pathways, whose function might merely be concealed by the loss of NF-κB, cannot be excluded. Their impact on mammary gland development would most likely be minor compared to effects brought about by NF-κB.

The presence of NF-κB in several stages of mammary gland morphogenesis is well established. Over the course of normal postnatal life, including pregnancy, lactation and involution, several NF-κB subunits and the main inhibitor, IκBα, are expressed in the mammary epithelium (Brantley et al., 2000;Cao and Karin, 2003;Clarkson and Watson, 1999). The mouse model for supressed NF-κB activity, which expresses non-degradable IκBα under the MMTV promoter, exhibits an early but transient delay in ductal branching during pregnancy (Demicco et al., 2005), whereas knock-in mice with a defective IKK complex display impaired alveologenesis (Cao et al., 2001;Demicco et al., 2005). Mice that lack the gene encoding for NF-κB suppressor *IκB*α (NF-κB gain-of-function) die perinatally. However, transplantation studies of the transgenic epithelium into wild type fat pads have demonstrated that increased NF-κB activity augments lateral ductal branching and hyperplasia (Brantley et al., 2001). Overall these and other studies highlight the importance of correct NF-κB pathway activity in mammary gland development and function.

NF-κB regulates growth, differentiation, and apoptosis in several tissues (Perkins, 2007). The high proliferation rates in K14-*Eda* mammary rudiments at the onset of branching morphogenesis would indicate that Eda/NF-κB exerts its effects through the increase of proliferation similarly to

NF-κB in postnatal development. Given the broad developmental roles and multiple target genes governed by NF-κB, it becomes clear that careful regulation of the transcription factor activity is necessary (Hayden MS 2006). Uncoupling NF-κB from normal regulation could contribute to tumorigenesis and metastasis (Shostak and Chariot, 2011). Indeed, NF-κB has been shown to be misregulated in several breast cancers. Numerous studies have reported an elevation of NF-κB activity in both mammary carcinoma cell lines and in different human breast cancer tissues (Cogswell et al., 2000;Nakshatri et al., 1997). Thus far Eda signalling has not been implicated in various breast cancers. However, an unpublished report described in a meeting abstract has associated enhanced Edar signalling with pregnancy-induced mammary tumours in mouse (Jobling et al., 2009). Whether misregulation of the pathway has a connection to breast cancer incidences will undoubtedly be uncovered by future studies.

4.4 Identification of Eda/NF-κB downstream targets in mammary primordia (II, III)

Transcriptional targets of *Eda* have thus far been analysed mainly in embryonic or adult skin (Cui et al., 2002;Cui et al., 2006;Fliniaux et al., 2008;Lefebvre et al., 2012;Mou et al., 2006), whereas genes regulated by Eda in mammary glands have remained largely unknown. We first set out to assess potent *Eda* target genes in the embryonic mammary bud with a candidate gene approach, which was based on a microarray, performed on genes differentially expressed in embryonic E14 $Eda^{-/-}$ back skin upon short exposure to recombinant Eda (Fliniaux et al., 2008;Lefebvre et al., 2012), as well as on other reports on Eda target genes in hair follicles (Zhang et al., 2009). These studies had implicated *PTHrP* and *Egfr* ligands *Areg* and *Epgn* as potent Eda target genes, and provided evidence of Wnt agonists *Wnt10a* and *Wnt10b* being NF-κB target genes in the hair placode.

We utilized a similar setup to induce upregulation of the Eda downstream targets in the mammary bud by exposing rudiments from E13.5 $Eda^{-/-}$ embryos to Fc-Eda. Quantitative RT-PCR (qRT-PCR) analysis of Fc-Eda treated mammary confirmed a significant increase in *Wnt10a*, *Wn10b* and *Pthrp* levels compared to untreated control samples, but only low-level upregulation of *Areg* and *Epgn. Dkk4* that has previously been identified as an Eda target gene (Fliniaux et al., 2008) was also highly upregulated in mammary primordia in response to Eda. Whole mount in situ hybridization (WMISH) analysis further showed that *Pthrp* and *Wnt10b* expression correlated with Eda levels in mammary primordia.

Next, we performed microarray profiling on E13.5 Fc-Eda treated (4h) $Eda^{-/-}$ mammary buds. The Eda treatment caused upregulation of 245 and downregulation of 78 genes. Among them were members of several distinct signalling pathway families such as Wnt, Fgf, Tnf, Tgf-β, chemokines and Hedgehog. In addition, adhesion molecules *Icam1* and *Madcam1*, metalloproteinases *Mmp9* and *Adamts-15*, and transcription factor *Foxi3* were induced by Eda. We validated the microarray results on selected upregulated genes with qRT–PCR and WMISH.

Transcription factor *Foxi3* was among the highly upregulated genes. It has previously been shown to be induced by Eda in developing hair follicles and teeth as well as transiently in mammary buds (Shirokova et al., 2013). Other upregulated genes were *Fgf20* and *Fgf17. Fgf20* has been identified as an Eda target gene in developing hair and teeth (Häärä et al., 2012;Huh et al., 2013). During mammogenesis, several *Fgf* genes are expressed in the mammary epithelium and/or in the adjacent epidermis (Eblaghie et al., 2004). Though mesenchymal Fgf10 and

epithelial receptor Fgfr2b have distinct roles in mammary induction (Mailleux et al., 2002), the importance of the other Fgfs is not yet well-understood and further studies will be required to uncover their precise roles. In addition to the previously identified Wnt pathway factors, we found Wnt inhibitor *Kremen2* and R-spondin receptor *Lgr4* to be among the genes regulated by Eda.

Even though qRT-PCR confirmed significant upregulation of *Adamts15*, *Icam1*, *Madcam1* and *Mmp9*, none of the factors were detected by WMISH in wild type mammary buds. However, Madcam1 and *Mmp9* were detected in K14-*Eda* primordia suggesting that they lie downstream of Eda in mammary buds. *Madcam1* and *Icam1* have been reported to be upregulated in the embryonic skin in response to Eda treatment (Lefebvre et al., 2012), but their possible significance to mammary gland development is unknown. Mmps have previously been linked to mammary gland branching morphogenesis (Simian et al., 2001). During embryonic mammogenesis, *ex vivo* inhibition of Mmp activity blocks ductal outgrowth and specifically Mmp2 has been shown to stimulate ductal growth (Hens et al., 2009). However, *Mmp9* deficiency does not impair mammary gland development (Wiseman et al., 2003), possibly due to redundancy with other Mmps.

Identification and successful validation of several distinct factors implies that Eda may act by tinkering the activity of multiple mammary-associated pathways during morphogenesis. Overall, our results were in line with previous findings that had identified Eda-induced genes in hair placodes. We found many shared genes between the analyses (Fliniaux et al., 2008;Lefebvre et al., 2012). Although these studies cannot be directly compared due to different microarray platforms used, it seems that the gene regulatory network governed by Eda is largely shared between hair follicles and mammary glands.

4.5 Eda upregulates Wnt activity which mediates its effects on mammary primordia (II, III)

Eda and Wnt signalling pathways have been shown to be intertwined during tooth, hair and salivary gland morphogenesis (Häärä et al., 2011;Mikkola, 2009), but their possible cooperative role in nascent mammary gland has not been explored. Since several components of the Wnt pathway were shown to be induced by Eda, and studies on hair placodes had proposed that one of the functions of Eda is to sustain canonical Wnt signalling activity (Zhang et al., 2009), we evaluated whether the Wnt pathway could be an important downstream effector of Eda signalling also in mammary morphogenesis.

We analysed expression patterns of many *Wnt* pathway genes that were upregulated in the microarray and had been validated by qRT-PCR. Our WMISH analysis revealed that several of them were upregulated in the supernumerary mammary-forming region early on. *Wnt10b, Lgr4, Krmn2* and additionally β-*cat* displayed a continuous streak of expression in the flank already at E12.5. The expression patterns were transient and only half a day later, the expression of these genes became confined to the emerging supernumerary placodes. We also studied the expression of other genes associated with placode formation (*Nrg3, Fgf2rb* and *Tbx3*). However, we were unable to establish any link between them and Eda signalling. To further test the effect of Eda on Wnt pathway activation, we turned to tissue culture. *Ex vivo* application of Fc-Eda to E12.5 TopGal reporter ventro-lateral skin explants induced an increase in reporter-positive cells between the third and the fourth mammary bud.

Next, we utilized the tissue culture setup to grow K14-*Eda* skin flanks that gave rise to supernumerary placodes similarly to *in vivo*. Introducing Wnt inhibitor XAV939 (Huang et al., 2009) to the culture system suppressed formation of the accessory structures in a dose-dependent manner. Higher concentrations fully inhibited supernumerary placode formation, whereas lower amounts of the molecule permitted some of the structures to emerge. Taken together, this indicates that Wnts are important effectors of Eda in supernumerary placode induction. Wnt activity has also previously been implicated in accessory mammary placode formation. Artificial activation of the pathway with LiCl gives rise to placode-like structures *ex vivo* (Chu et al., 2004).

During mammary primordia development, *TopGal* reporter activity is found along the milk line from E10.5 onwards (Chu et al., 2004). It thus precedes NF-κB activity that is absent from the mammary-forming region prior to E11. Our results show that even though Eda is not originally required for the induction of Wnts, later on its levels modulate expressions of several individual Wnt pathway factors. However, Wnt pathway activity or maintenance is not solely dependent on Eda. Taken together, this places Wnts upstream of Eda signalling in mammary induction. After the placodes have formed, Eda potentiates and modulates Wnt activity by upregulating the activators and inhibitors of the pathway. Upregulation of Wnt inhibitors suggests a presence of a negative feedback signalling. This could be a means for regulating placode size and to ensure that the primordia do not develop too close to each other. In conclusion, Eda-dependent increase in Wnt signalling activity, most likely via upregulation of multiple Wnt pathway genes, is the likely cause for induction of supernumerary mammary placodes in K14-*Eda* mice.

We also considered whether Eda-induced upregulation of *Wnt* agonists could explain the enhanced ductal development of K14-*Eda* mice. Indeed, it has been shown that transgenic overexpression of *Wnt10b* under MMTV-promoter results in a hyperbranched ductal phenotype in adult animals (Lane and Leder, 1997). On the contrary, genetic ablation of *Lgr4* impairs adult branching morphogenesis (Wang et al., 2013). Similarly, mutations of *Lrp5* and *Pygopus2* that decrease Wnt signalling output result in stunted ductal phenotypes during embryogenesis. We analysed *Wnt10b* expression by *in situ* hybridization, which showed Eda-dependent correlation at the onset of branching morphogenesis. We also compared *TopGal* and *BatGal* Wnt reporter expressions at the onset and during branching morphogenesis between wild type, K14-*Eda* and *Eda* null mammary primordia. Curiously, reporter expression appeared to be downregulated in the K14-*Eda* mammary epithelium but upregulated in the mesenchyme. Moreover, immunohistochemical analysis of Lef1 exhibited similar correlation. As Eda upregulates both Wnt agonists and antagonists, the detected opposing effects of Eda in the epithelium and the mesenchyme could reflect the function of these different factors in separate tissue compartments. Conclusions should, however, take into account that the K14-*Eda* glands exhibit a more advanced ductal phenotype. Some of the observed differences might be a reflection of normal differences between morphological stages.

We also assessed the effects of increased Wnt activity in tissue culture. However, *ex vivo* activation of the Wnt pathway with Wnt3a recombinant protein was not able to compensate for the lack of Eda and rescue the impaired ductal phenotype of *Eda* null mammary rudiments. Nevertheless, Wnt3a did increase ductal branching in wild type primordia. Most likely the accelerated branching witnessed in K14-*Eda* background is a consequence of combinatory effects of Wnt signalling and some other Eda induced factor(s). These may include PTHrP and Egfr agonists, which all accelerated ductal growth and branching of wild-type mammary buds *ex vivo*.

4.6 Overexpression of Eda leads to loss of sexual dimorphism in the mammary glands (III)

In mice the sexual dimorphism of the mammary glands occurs during embryogenesis due to AR-mediated apoptosis at E14 (Dunbar et al., 1999;Kratochwil and Schwartz, 1976;Kratochwil, 1977). Unexpectedly, we found that the K14-*Eda* males possess mammary glands and nipples even in adulthood. Examination of the K14-*Eda* male mammary primordia at E18 revealed a clearly more advanced ductal morphogenesis compared to wild type female glands and a very close resemblance to K14-*Eda* female rudiments. We also observed a similar situation in 3-week-old prepubertal animals. The differences between K14-*Eda* female and male glands only appeared at the onset of puberty. The oestrogen-mediated growth burst of the glands occurred in the females but was never witnessed in the male mice. These observations indicate that the hormonal control of the gland development is most likely preserved. Expectedly, ductal structures were absent or severely stunted in control males.

At E15.5, the stalk region of the K14-*Eda* male mammary bulb exhibited an extremely narrowed morphology due to mesenchymal condensation which is typical for wild type males of a similar stage. Immunohistochemical analysis revealed no abnormalities in the mesenchymal AR expression or apoptosis of the epithelial stalk in K14-*Eda* males. A probable explanation for the uninterrupted epithelial morphogenesis in the animals is the precocious and more advanced ductal development compared to the wild type. At E14, the wild type rudiment is still at the bulb stage, whereas the K14-*Eda* gland has already invaded through the AR-positive primary mammary mesenchyme into the fat pad precursor, AR free, compartment. Most likely these ductal tips are able to escape the androgen-mediated apoptosis and continue their development as blind ducts that have a severed connection to the upper epidermis.

Formation of the nipple sheath, which gives rise to the nipple, is thought to be dependent on cues originating from the mammary mesenchyme (Dunbar et al., 1998). Curiously, the apoptotic destruction of the mammary mesenchyme does not interfere with nipple sheath development, as K14-*Eda* males retain nipples. This indicates that the mammary mesenchyme must remain intact in K14-*Eda* males at least to some extent. Perhaps the amount of the specialized mesenchymal tissue in K14-*Eda* embryos is higher than in the wild type counterparts, and the androgen-mediated apoptosis does not destroy it completely. As the K14-*Eda* glands represent a more advanced developmental stage already at E14, it is also possible that the mammary mesenchyme has had sufficient time to instruct the nipple sheath to form by E14. However, additional studies would be required to verify the theory.

These results demonstrate that during embryogenesis the mammary microenvironment is very similar between male and female mice. Male glands are able to continue their development as long as they manage to reach the secondary mammary mesenchyme. The epithelial-mesenchymal interactions between the ductal tips and the fat pad precursor cells obviously are intact, as branching morphogenesis takes place even in the blind ducts of K14-*Eda* males.

Loss of sexual dimorphism has also been described in a few other mouse strains. Pthrp null mice maintain mammary gland development beyond the E14 stage. AR expression in the primary mammary mesenchyme is dependent on Pthrp signalling, and in its absence the androgen-mediated receptor activation and following apoptosis in the primordia never takes place (Dunbar et al., 1999). A similar situation occurs in *Gli3*$^{-/-}$mice in which AR expression is diminished and the males of the strain retain mammary sprouts to some extent (Chandramouli et al., 2013). Transgenic male mice that express human aromatase enzyme have abnormally low

testosterone and high oestrogen levels. The hormonal imbalance induces ductal development also in the male mice (Li et al., 2002).

These circumstances, however, differ from the one observed in K14-*Eda* males that have normal AR expression and activation. A previous report has shown that the *Wnt10b* gain-of-function mouse strain exhibits mammary development that involves highly-branched mammary ducts in both genders. This implies that the increased *Wnt10b* levels enable the male mammary rudiments to bypass the embryonic destruction of the organs. A similar phenotype is also observed in *Wnt1* transgenic males (Lane and Leder, 1997;Tsukamoto et al., 1988). We have identified *Wnt10b* as one of the potent downstream targets of Eda signalling and shown that ectopic activation of the Wnt pathway increases and/or accelerates embryonic ductal morphogenesis *ex vivo.* It is thus possible that the phenotypic similarities between the K14-*Eda* and the above-described Wnt gain-of-function strains originate from shared developmental characteristics.

Why males of certain mammalian species retain nipples and mammary glands, whereas in others development is supressed already during embryogenesis, is not well understood. However, in all mammals the gland formation commences, as a default developmental program, before the gender-specific characteristics emerge. Most likely the importance of mammary glands for future offspring survival is so vital, whereas the presence of unnecessary glandular structure is so insignificant, that there has not been high enough selective pressure to erase them from males.

5 Concluding remarks

This thesis work has focused on elucidating the role of Eda in the prepubertal mammary gland development. We found that Eda has a dual role in mammogenesis. First, Eda induces supernumerary mammary placode formation within the milk line (Mustonen et al., 2003) and in the neck, an area that has not previously been described to possess mammary induction capacity. Second, branching morphogenesis of the nascent gland correlates with the amount of Eda. Whether the Eda-dependent alterations in ductal morphogenesis are retained during hormonal development was not examined. However, a recent study of adult *Edar* gain-of-function and *Eda-/-* and *Edar-/-* mice would suggest that Eda levels affect branching morphogenesis also during later, postnatal stages of development or that the morphological differences originating from embryogenesis are sustained (Chang et al., 2009).

Transcription factor NF-κB is thought to be a major mediator of Eda signalling (Mikkola, 2009). We showed that NF-κB activity contributes to prepubertal branching morphogenesis and is crucial for Eda-induced accessory placode formation and accelerated branching morphogenesis. This indicates that NF-κB is the main downstream transducer of Eda signalling also in the mammary gland. We identified several potent transcriptional targets of Eda/NF-κB signalling. Some of these genes had previously been shown to be regulated by Eda/NF-κB in other ectodermal organs (Lefebvre et al., 2012;Zhang et al., 2009). This indicates that genetic networks governed by Eda are shared between different ectodermal organs at least to some extent. We discovered that Wnt/β-cat signalling is an important downstream effector of Eda/NF-κB. The formation of supernumerary mammary placodes and accelerated ductal development in wild type primordia were dependent on the pathway activity. However, other factor(s) are bound to be involved in mediating Eda's effects in prepubertal mammary gland.

Considering the variety in the number of mammary glands present in different mammals, one might speculate that modulation of the Eda pathway could be an evolutionary means to increase or restrict the number of the organs. The K14-Eda mice display a curious likeness for example to the multimammate mouse *(Mastomys)* that have equally spaced nipples along the ventral surface from the axilla to inguinal region. Formation of supernumerary nipples is fairly common in humans and other studied mammals (Kajava, 1915). Misregulation of the Eda pathway could account for at least some of these malformations.

Mammary gland defects associated with XLHED and autosomal HED are only now being characterized more intensively. Our results suggest that the glandular tissue of the breast could be defective in the affected patients, as the preliminary NFED report appears to indicate. Our results suggest that the defects arise already during neonatal development. However, whether Eda has an effect on pregnancy-induced branching, a prerequisite for successful lactation is still unknown. It is also possible that the defective lactation is a combination of embryonic defects and impaired ductal development during pregnancy. NF-κB activity during the hormone-dependent morphogenesis is well documented, and it would be interesting to find out whether Eda mediates some of the effects.

To conclude, the mammary gland is an interesting experimental system for several reasons. The embryonic development offers possibilities to study various important phenomena including organ induction, epithelial-mesenchymal interactions, ductal invasion and branching morphogenesis in addition to associated cellular mechanisms, such as cell proliferation and migration. The described *ex vivo* methods for culturing mammary primordia enable the

evaluation of individual proteins, inhibitors or other small molecules during different stages of mammary gland development. In both procedures described, mammary buds are retained in their original locations, which could also enable assessment of the stromal microenvironment. *Ex vivo* culture of explants harbouring fluorescently labelled mammary cells should permit live-imaging of the tissue even at a single-cell resolution, as previously shown for e.g. developing hair follicles (Ahtiainen et al., 2014). This approach is bound to reveal many details about embryonic mammogenesis. Considering the high prevalence of breast cancer and the parallel characteristics between embryonic mammary gland development and breast tumorigenesis, understanding the cellular and morphological mechanisms that control normal ductal development and microenvironment is increasingly important.

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